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(54) **Method for identifying and characterizing organisms.**

(57) A method of characterizing an unknown organism species comprises determining the position of part or whole of evolutionarily conserved sequences in genetic material of said organism, relative to a known position of restriction endonuclease cleavage sites in said genetic material (other than by determining the chromatographic pattern of retriction endonuclease digested DNA from said unknown organism, which digested DNA has been hybridized or reassociated with ribosomal RNA information containing nucleic acid from or derived from a known probe organism), thereby to obtain an identifying genetic characterization of said unknown organism, and comparing said characterization with information from at least two sets of identifying genetic characterizations derived from the same conserved sequences, each of said sets defining a known organism species.

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METHOD FOR IDENTIFYING AND CHARACTERIZING ORGANISMS

The present invention relates to a method for the rapid and accurate characterization and identification of organisms, including prokaryotic and eukaryotic organisms, such as bacteria, plants, and animals.

The classification of living organisms has traditionally been done along more or less arbitrary and somewhat artificial lines. For example, the living world has been divided into two kingdoms: Plantae (plants) and Animalia (animals). This classification works well for generally familiar organisms, but becomes difficult for such organisms as unicellular ones (e.g., green flagellates, bacteria, blue-green algae), since these differ in fundamental ways from the "plants" and "animals".

It has been suggested to simply divide organisms with respect to the internal architecture of the cell. In this scheme, all cellular organisms are either prokaryotic or eukaryotic. Prokaryotes are less complex than eukaryotes, they lack internal compart-

mentalization by unit membrane systems, and lack a defined nucleus. Prokaryotic genetic information is carried in the cytoplasm on double-stranded, circular DNA; no other DNA is present in cells (except for the possible presence of phage, bacterial viruses, and cirular DNA plasmids, capable of autonomous replication). Eukaryotes, on the other hand, have a multiplicity of unit membrane systems which serve to segregate many of the functional components into specialized and isolated regions. For example, genetic information (DNA) can be found in a well-compartmentalized nucleus and also in organelles: mitochondria and (in photosynthetic organisms) chloroplasts. The replication, transcription, and translation of the eukaryotic genome occurs at either two or three distinct sites within the cell: in the nucleocytoplasmic region, in the mitochondrion, and in the chloroplast.

The differences between prokaryotes and eukaryotes, however, breaks down when a comparison of mitochondria and chloroplasts is carried out with prokaryotes: these organelles are today considered to have been derived from free-living prokaryotes, which entered into an endosymbiotic relation with primitive eukaryotes, and eventually became closely integrated with the machinery of the host cell and incapable of independent existence (see e.g., Fox, G.E. et

al, Science 209:457-463 (1980), at 462; Stanier, R. Y. et al, "The Microbial World," Fourth Edition, Prentice-Hall, Inc., 1976, at p. 86). For example, it has been demonstrated that DNA from mouse L cell mitochondria carrying the ribosomal RNA gene region exhibits notable sequence homologies to *Escherichia coli* ribosomal RNA, thus providing strong support for the endosymbiotic model (Van Etten, R. A. et al, Cell, 22:157-170 (1980)). It has also been shown that the nucleotide sequence of 23S ribosomal DNA from *Zea mays* chloroplast has 71% homology with 23S ribosomal DNA from *E. coli* (Edwards, K. and Kossel, H., Nucleic Acids Research, 9:2853-2869 (1981)); other related work (Bonen, L. and Gray, M. W., ibid, 8:319-335 (1980)) also further supports the general concept.

In this model the eukaryotic cell is a phylogenetic "chimera" with organelle components that are clearly prokaryotic in nature. The "prokaryotic-eukaryotic" dichotomy then, also has drawbacks, even as a broad classification method.

Where classification of organisms becomes more than a scientific exercise is in the identification of plants and animals for hybridization and breeding purposes, and in the accurate and reliable identification of microorganisms which may infect so-called "higher" organisms or other media. For example, the plant-breeder, cattle breeder, or fish breeder may wish to

have a quick and reliable means of identifying different species and strains of their subjects. The veterinarian, physician, or horticulturist may wish to have an accurate identification of any infectious organisms (parasites, fungi, bacteria, etc.) and viruses present in examined plant or animal tissues. The correct identification of species of these organisms and viruses is of particular importance.

The problem can best be illustrated by referring to the identification of bacteria. Names of bacterial species usually represent many strains, and a strain is considered to be a population derived from a single cell. Bacterial species are usually defined by describing the degree of homogeneity and diversity of attributes in representative samples of strains of species. Precise definitions of bacterial species are difficult to express because subjective limits to strain diversity within species are required to define species boundaries. (Buchanan, R. E., International Bulletin of Bacteriological Nomenclature and Taxonomy, 15:25-32 (1965)). The practical application of definitions of species to the identification of an unknown bacterial strain requires the selection of relevant probes, such as substrates and conditions to detect phenotypic attributes, and radioactively-labeled DNA from the same species. Because of the diversity of bacterial species, a screening procedure is the primary

tool used in the classical, progressive method for identification of a strain. Results of the screening procedure are then used to predict which other laboratory methods and reagents are relevant for definitive identification of the strain. Identification is ultimately based on certain phenotypic and genotypic similarities between the unidentified strain and characterized species. The challenge is to precisely define the boundaries of species, preferably in terms of a standard probe which reveals species-specific information, so that definitions of species can be directly and equally applied to the identification of unknown strains.

Bergey's Manual of Determinative Bacteriology (Buchanan, R. E. and Gibbons, N. E., Editors, 1974, 8th Edition, The Williams & Wilkins Company, Baltimore) provides the most comprehensive treatment of bacterial classification particularly for nomenclature, type strains, pertinent literature, and the like. It is, however, only a starting point for the identification of any species since, *inter alia*, it is normally out of date, and is limited in space to describing species quite briefly. (See for example, Brenner, D. J., "Manual of Clinical Microbiology," 3rd Edition, American Society of Microbiology, Washington, D.C., 1980, pages 1-6.)

The term "species", as applied to bacteria, has been defined as a distinct kind of organism, having certain distinguishing features, and as a group of organisms which generally bear a close resemblance to one another in the more essential features of their organization. The problem with these definitions is that they are subjective; Brenner, supra, at page 2. Species have also been defined solely on the basis of criteria such as host range, pathogenicity, ability or inability to produce gas in the fermentation of a given sugar, and rapid or delayed fermentation of sugars.

In the 1960's, numerical bacterial taxonomy (also called computer or phenetic taxonomy) became widely used. Numerical taxonomy is based on an examination of as much of the organism's genetic potential as possible. By classifying on the basis of a large number of characteristics, it is possible to form groups of strains with a stated degree of similarity and consider them species. Tests which are valuable for the characterization of one species, however, may not be useful for the next, so this means to define species is not directly and practically applicable to the identification of unknown strains. Although this may be overcome in part by selecting attributes which seem to be species specific, when these attributes are used to identify unknown strains, the species definition is applied indirectly. See for example Brenner, supra, at

pages 2-6. The general method, furthermore, suffers from several problems when it is used as the sole basis for defining a species, among them the number and nature of the tests to be used, whether the tests should be weighted and how, what level of similarity should be chosen to reflect relatedness, whether the same level of similarities is applicable to all groups, etc.

Hugh, R. H. and Giliardi, G. L., "Manual of Clinical Microbiology," 2nd Edition, American Society for Microbiology, Washington, D.C., 1974, pages 250-269, list minimal phenotypic characters as a means to define bacterial species that makes use of fractions of genomes. By studying a large, randomly selected sample of strains of a species, the attributes most highly conserved or common to a vast majority of the strains can be selected to define the species. The use of minimal characters is progressive and begins with a screening procedure to presumptively identify a strain, so that the appropriate additional media can be selected. Then the known conserved attributes of the species are studied with the expectation that the strain will have most of the minimal characters. Some of the minimal characters do not occur in all strains of the species. A related concept is the comparative study of the type, the neo-type, or a recognized reference strain of the species. This control is neces-

sary because media and procedures may differ among laboratories, and it is the strain, not the procedure, that is the standard for the species.

A molecular approach to bacterial classification is to compare two genomes by DNA-DNA reassociation. A genetic definition of species includes the provision that strains of species are 70% or more related. With DNA-DNA reassociation a strain can be identified only if the radioactively labeled DNA probe and unknown DNA are from the same species. The practical application of this 70% species definition however is limited by selection of an appropriate probe. This may be overcome in part by selecting phenotypic attributes which seem to correlate with the reassociation group, but when these are used alone the DNA-DNA reassociation species definition is also applied indirectly.

Brenner, supra, at page 3, states that

> the ideal means of identifying bacterial species would be a 'black box' which would separate genes, and instantly compare the nucleic acid sequences in a given strain with a standard pattern for every known species-something akin to mass spectrophotometric analysis.

Brenner, however, concedes that although restriction endonuclease analysis can be done to determine common sequences in isolated genes, "we are not at all close to

having an appropriate black box, especially one suited for clinical laboratory use." His words could be equally applied to any species of organism.

This brief review of the prior art leads to the conclusion that there presently exists a need for a rapid, accurate, and reliable means for identifying unknown bacteria and other organisms, and to quickly classify the same, especially to identify the organism of a disease, or of a desirable biochemical reaction. The method should be generally and readily useful in clinical laboratories, should not be dependent on the number of tests done, on the subject prejudices of the clinician, nor the fortuitous or unfortuitous trial and error methods of the past. Further, a need also exists for a method useful for identifying and distinguishing genera and species of <u>any</u> living organism, which can be readily and reliably used by veterinarians, plant-breeders, toxicologists, animal breeders, entomologists and in other related areas, where such identification is necessary.

It is therefore an object of the invention to provide a method which may be found to be quick, reliable and accurate of objectively identifying organisms, especially - but not limited to - microorganisms.

Yet another object of the invention is to provide a method of identifying organisms such as bacteria which utilizes the organisms' genome.

Another object of the invention is to provide a method of characterizing and identifying species and

genera of pathogenic organisms in the clinical laboratory, so as to provide the capability of characterizing and identifying the cause of any given animal or plant disease.

Still another object of the invention is to provide various products useful in the aforementioned methodologies.

These and other objects of the invention, as will hereinafter become more readily apparent, have been attained by providing:

A method of characterizing an unknown organism species which comprises determining the position of part or whole of evolutionarily conserved sequences in genetic material of said organism, relative to a known position of restriction endonuclease cleavage sites in said genetic material (other than by determining the chromatographic pattern of restriction endonuclease digested DNA from said unknown organism, which digested DNA has been hybridized or reassociated with ribosomal RNA information containing nucleic acid from or derived from a known probe organism), thereby to obtain an identifying genetic characterization of said unknown organism, and comparing said characterization with information from at least two sets of identifying genetic characterizations derived from the same conserved sequences, each of said sets defining a known organism species.

Still another object of the invention has been attained by providing:

A method of diagnosing a pathogenic organism infection in a sample which comprises identifying the organism in said sample by the aforementioned method.

For a better understanding of the invention, and to show how it may be put into effect, reference will now be made, by way of example, to the accompanying drawings in which:

FIGURE 1 shows the EcoR I restriction endonuclease digest of DNA isolated from strains of *Pseudomonas aeruginosa*, using cDNA to 16S and 23S ribosomal RNA (rRNA) of *E. coli* as the probe.

FIGURE 2 shows the Pst I restriction endonuclease digest of DNA isolated from strains of *P. aeruginosa*, using cDNA to 16S and 23S rRNA of *E. coli* as the probe.

FIGURE 3 shows the EcoR I restriction endonuclease digest of DNA isolated from species of glucose-nonfermenting, gram-negative rods, using cDNA to 16S and 23S rRNA of *E. coli* as the probe.

FIGURE 4 shows the Pst I restriction endonuclease digest of DNA isolated from species of glucose-nonfermenting, gram-negative rods using cDNA to 16S and 23S rRNA of *E. coli* as the probe.

FIGURE 5 shows the EcoR I restriction endonuclease digest of DNA isolated from various *Bacillus subtilis* strains, using cDNA to 16S and 23S rRNA of *E. coli* as the probe.

FIGURE 6 shows the Pst I data for the same strains as in FIGURE 5, with the same probe.

FIGURE 7 shows the Bgl II data for the same strains as in FIGURES 5 and 6, with the same probe.

FIGURE 8 shows the Sac I data for the same strains as in FIGURES 5-7, with the same probe.

FIGURE 9 shows the EcoR I restriction endonuclease digest of DNA isolated from *B. subtilis* and *B. polymyxa*, using cDNA to 16S and 23S rRNA from *E. coli* as the probe.

FIGURE 10 shows the Pst I data for the same strains as in FIGURE 9 with the same probe.

FIGURE 11 shows the Bgl II and Sac I data for the same strains as in FIGURES 9 and 10, with the same probe.

FIGURE 12 shows the detection of *Streptococcus pneumoniae* in EcoR I digested DNA from infected mouse tissues using cDNA from 16S and 23S rRNA from *E. coli* as the probe.

FIGURE 13 shows the identification of a mouse species by comparing Pst I digests of DNA isolated from mammalian tissues, using cDNA to 18S and 28S rRNA from cytoplasmic ribosomes of *Mus musculus domesticus* (mouse).

FIGURE 14 shows the EcoR I digested DNA from mouse and cat tissues hybridized with *Mus musculus domesticus* 28S rRNA cDNA probe.

FIGURE 15 shows Sac I digested DNA from mammalian tissues hybridized with *Mus musculus domesticus* 18S and 28S rRNA cDNA probe.

FIGURE 16 shows EcoR I digested DNA from mammalian tissues and cell cultures hybridized with *Mus musculus domesticus* 18S and 28S rRNA cDNA probe.

This invention is based on the inventor's realization that, if species are discrete clusters of strains related to a common speciation event, there should be, despite divergence, a likeness shared by strains that objectively defines the species boundary; strains of species should contain structural information which is a clue to their common origin. The greatest amount of an organism's past history survives in semantides, DNA and RNA, (Zuckerkandle, E. and Pauling, L., Journal of Theoretical Biology, 8:357-366 (1965)).

In European Patent Application (EP-A-) No.0076123 (herein incorporated by reference) the inventor described a system for the definition of species and characterization of organisms which makes use of information contained in ribosomal RNA genes (rRNA). Ribosomal RNA has a structural and functional role in protein synthesis (Schaup, Journal of Theoretical Biology, 70:215-224 (1978)), and the general conclusion from rRNA-DNA hybridization studies, is that the base sequences of ribosomal RNA genes are less likely to change, or are more conserved during evolution, than are the majority of other genes (Moore, R. L., Current Topics In Microbiology and Immunobiology, Vol. 64:105-128 (1974), Springer-Verlag, New York). For example, the primary structure of 16S rRNA from a number of bacterial species has been inferred from oligonucleotide analysis

(Fox, G. E. et al, International Journal of Systematic Bacteriology, 27: 44-57 (1977)). There are negligible differences in the 16S oligomer catalogs of several strains of E. coli (Uchida, T. et al, Journal of Molecular Evolution, 3:63-77 (1974)); the substantial differences among species, however, can be used for a scheme of bacterial phylogeny (Fox, G.E., Science, 209:457-463 (1980)). Different strains of a bacterial species are not necessarily identical; restriction enzyme maps show that different EcoR I sites occur in rRNA genes in two strains of E. coli (Boros, I.A. et al, Nucleic Acids Research 6:1817-1830 (1979)). Bacteria appear to share conserved rRNA gene sequences and the other sequences are variable (Fox, 1977, supra).

The present inventor had thus discovered that restriction endonuclease digests of DNA have sets of fragments containing conserved sequences that are similar in strains of a species of organism (e.g., bacteria), but different in strains of other species of the organism; i.e., despite strain variation, enzyme specific sets of restriction fragments with high frequencies of occurrence, minimal genotypic characters, define the species. This is the essence of the invention described in EP-A-0076123 and also that of the invention described herein.

The present invention constitutes an extension of the concepts developed in EP-A-0076123, in that

it has further been discovered that there exist sequences, in addition to those of rRNA, which are highly conserved through evolution and which may be as useful as rRNA sequences in the identification system. In other words, the present invention provides means for carrying out the identification and characterization techniques of EP-A-0076123, using any probe which is conserved, other than rRNA. The present invention also provides additional examples of methods which may be used in the identification process. The present inventor has also discovered that the method is general, in that it is applicable to both eukaryotic and prokaryotic DNA, using a conserved nucleic acid probe from any organism, prokaryotic or eukaryotic, of the same or different (classic) taxonomic classification than the organism being identified.

The invention offers an objective method of defining organisms based on conserved sequences of DNA or other genetic material in relation to known positions such as restriction endonuclease sites. The detection of restriction fragments containing a conserved sequence may be carried out by hybridizing or reassociating DNA segments with nucleic acid containing conserved sequence information from a probe organism.

By the "organism" which can be characterized (which term is meant to include "identified") by the process of the invention, it is meant to include virtually any organism which, by definition, contains DNA or RNA in its genome.

In this respect it is useful to refer to a classical taxonomic scheme as a point of reference.

All organisms belonging to the Kingdoms Monera, Plantae and Animalia are included. For example, among those of the Kingdom Monera can be mentioned the Schizomycetes (Bacteria) of the classes myxobacteria, spirochetes, eubacteria, rickettsiae, and the cyanopytha (blue green algae). Among those of the Kingdom Plantae can be mentioned the Division Euglenophyta (Euglenoids), Division Chlorophyta (green-algae) classes chlorophyceae and charophyceae, Division Chrysophyta, classes xanthophyceae; chrysophyseae, bacillariophyceae; Division Pyrrophyta (Dinoflagellates); Division Phaeophyta (Brown algae); Division Rhodophta (Red algae); Division Myxomycophyta (slime molds), classes myxomycetes, acrasiae, plasmodiophoreae, labyrinthuleae; Division Eumycophyta (true fungi), classes phycomycetes, ascomycetes, and basidomycetes; Division Bryophta, classes hepaticae, anthocerotae, and musci; Division Tracheophyta (Vascular plants), subdivisions psilopsida, lycopsyda, sphenopsida, pteropsida, spermopsida classes cycadae, ginkgoae, coniferae, gneteae and angiospermae subclasses dicotyledoneae, monocotyloedoneae. Among those of the Kingdom Animalia can be mentioned the Subkingdom Protozoa, Phylum Protozoa (Acellular animals) subphylum plasmodroma, classes flagellata, sarcodina and sporozoa; subphylum ciliophora, class ciliata; the Subkingdom

Parazoa, Phylum porifera (Sponges), class calcarea, hexactinellida, and desmospongiae; the Subkingdom Mesozoa, Phylum mesozoa; the Subkingdom Metazoa, Section Radiata, Phylum coelenterata, classes hydrozoa, scyphozoa, anthozoa, Phylum ctenophora, classes tentaculata and nuda; Section Protostomia Phylum platyhelmintes (flatworms) classes tubellana, trematoda, and cestoda; Phylum nemertina; Phylum acanthocephala; Phylum aschelmintles, classes rotifera, gastrotricha, kinorhyncha, priapulida, nematoda and nematomorpha; Phylum entoprocta; Phylum ectoprocta, classes gymnolaemata and phylactolaemata; Phylum phoronida; Phylum braciopoda, classes inarticulata and articulata; Phylum mollusca (molluscs) classes amphineura, monoplacophora, gastropoda, scaphopoda, pelecypoda, and cephalopoda; Phylum sipunculida; Phylum echiurida; Phylum annelida, classes polychaeta, oligochaeta and hirudinea; Phylum onychophora; Phylum tardigrada; Phylum pentastomida; Phylum arthropoda, subphylum trylobita, subphylum chelicerata classes xiphosura, arachmida, pycnogomida, subphylum mandibulata classes crustacea, chilopoda, diplopoda, pauropoda, symphyla, insecta of the orders collembola, protura, diplura, thysanura, ephemerida, odonata, orthoptera, dermaptera, embiania, plecoptera, zoraptera, corrodentia, mallophaga, anoplura, thysasnoptera, hemiptera, neuroptera, coleoptera, hymenoptera, mecoptera, siphonaptera, diptera, trichoptera and lepidoptera; those of the

Section Deuterostomia, phylum chaetognatha, phylum echinodermata, classes crinoidea, asterordea, ophiuroidea, echinoidea, and holoturoidea, phylum pogonophora; phylum hemichordata, classes enteropneusta, and pterobranchia; phylum chordata, subphylum urochordata, classes ascidiaciae, thaliaceae, larvacea; subphylum cephalochordata, subphylum vertebrata, classes agnatha, chondrichthyes, osteichthyes (subclass saccopteiygii orders crossopterygii and dipnoi), amphibia, repitilia, aves and mammalia, subclass prototheria, subclass theria, orders marsupialia, insectivora, dermoptera, chiroptera, primates, edentata, pholidota, lagomorpha, rodentia, cetaceae, carnivora, tubulidentata, probosicdea, hyracoidea, sirenia, perissodactyla and artiodactyla.

It is understood that beyond the order, the organisms are still classified according to their families, tribes, genus and species, and even subspecies, infrasubspecific taxons, and strains or individuals. In addition, cell cultures (plant or animal), as well as viruses can also be identified. These classifications are used in this application for illustrative purposes only, and are not to be taken as exclusive. The organism is either known or unknown, most commonly the organism is an unknown being identified.

Functionally, for the purposes of this invention, it is convenient to divide all organisms into the eukaryotes and the prokaryotes. When identifying a prokaryotic

organism, the DNA to be analyzed is that present in the cells or in the non-compartmentalized chromosomes. When identifying a eukaryotic organism one may either use the nuclear DNA or the organelle DNA (mitochondrial DNA or chloroplast DNA).

Briefly, high molecular weight DNA and/or small circular DNAs are isolated from the organism to be identified in order to analyze the conserved sequences (and possibly sequences that could be used to create a taxon below the rank of species or infrasubspecific subdivisions.) The DNA's are extracted by methods which are well-known to the art.

The DNA's are analyzed to ascertain both 1) the presence and position of the conserved sequences and 2) their position relative to endonuclease restriction sites. The easiest way to analyze for the presence of the conserved sequences is to utilize a polynucleotide probe capable of hybridizing with the conserved DNA sequence. However, direct sequence information as obtained by chemical sequence determination and analysis thereof could also be utilized. In EP-A-0076123 the probe utilized was an rRNA information containing-probe; in this case any other probe having conserved sequences could be used. In an analogous manner, the easiest way of finding a given set of endonuclease restriction sites is to cleave the DNA with the appropriate restriction enzymes. (This, indeed, is the manner taught and

practised in EP-A-0076123. However, alternative methods, such as sequence information coupled with known restriction site sequences, or cleavage and partial sequencing could also be used.

Most commonly DNA's are going to be cut at specific sites into fragments by restriction endonucleases. The fragments are separated according to size by a chromatographic system. In EP-A-0076123 gel chromatography was used as an example of a useful chromatographic system. However, other systems can also be used, such as high pressure liquid chromatography, capillary zone electrophoresis, or other separation techniques. In using gel chromatography, the fragments are separated, the gels are stained, as is otherwise well-known in the art, and standardized as to the fragment sizes using standards curves constructed with fragments of known sizes. The separated fragments may then be transferred to cellulose nitrite paper by the Southern blot technique (Southern, E. M., Journal of Molecular Biology, 38:503-517 (1975), herein incorporated by reference), and covalently bound thereto by heating. The fragments containing the conserved sequences are then located by their capacity to hybridize with a nucleic acid probe containing conserved sequence information. Alternatively, hybridization can occur after digestion but before separation; or restriction cleavage can occur after hybridization, followed by separation of the

fragments.

The nucleic acid probe can either be non-radioactively labeled or, preferably, radioactively labeled. When radioactively labeled, the probe can be RNA, or preferably DNA which is complementary to RNA (cDNA), either synthesized by reverse transcription or contained on a cloned-fragment, which can be labeled, for example, by nick translation. Also, synthetic oligodeoxyribonucleotides may be prepared with labeled nucleotides.

The well-defined probe is derived from an arbitrarily chosen organism, see infra, or may be a consensus sequence. Once hybridization has occurred, the hybridized fragments are detected by selectively detecting double stranded nucleic acid (non-radiolabeled probe), or visualized by, e.g., autoradiography (radio-labeled probe). The size of each fragment which has been hybridized is relative to the restriction sites and is determined from the distance traveled using standard curves, as described previously. The amount of hybridization, the pattern of hybridization, and the sizes of the hybridized fragments, which are relative to restriction sites, can be used individually or in conjunction to identify the organism.

The genetic characterization that emerges from this technique can be readily compared to equivalent characterizations derived from at least two and up to a

multiplicity of known, standard organisms, genera or species. After a preliminary broad classification has already been carried out (using, for example, classical taxonomy), the comparison can be either by visual inspection and matching of appropriate chromatographic patterns, (as in EP-A-0076123) by comparison of hybridized restriction fragment sizes, by band intensity (amount of hybridization) or by any combination thereof. Ideally, the comparison is carried out with a one-dimensional computer-based pattern recognition system, such as those used in point-of-sale transactions.

The present inventor discovered that when using the aforementioned method, the genetic characterizations for organisms of the same species are substantially similar, with minor variations allowed for intraspecies differences due to strain variations, whereas differences between species, and differences between genera (and higher classifications) are maximal.

The use of enzyme-specific fragment variations among strains of a species permits the typing of strains for various purposes; e.g. in the case of bacteria, for epidemiological purposes. In fact, restriction enzymes can be chosen for their ability to distinguish strains within species.

The "probe organism" used in the present invention, and from which is obtained the nucleic acid probe, can also be any of the aforementioned organisms; it can be

either eukaryotic or prokaryotic. The only limitation is given by the fact that the conserved sequence-containing probe should hybridize maximally with the unknown organism's DNA.

There are four types of conserved sequence information-containing probes: 1) prokaryotic probes (especially bacterial-derived), 2) eukaryotic mitochondrial probes, 3) eukaryotic chloroplast probes, and 4) eukaryotic non-organelle probes. There are also four sources of DNA (to be endonuclease digested): 1) prokaryotic cellular DNA, 2) eukaryotic mitochondrial DNA, 3) eukaryotic chloroplast DNA, and 4) eukaryotic nuclear DNA. The following hybridization table can thus be constructed (Table 1).

Table 1

Hybridization Table

Unknown organism DNA	Conserved Gene Sequence Probe			
	Prokaryotic	Eukaryotic		
		Mito-chondrial	Chloro plast	Non-organelle
Prokaryotic	+	+	+	-
Eu.(1) Mitochondria	+	+	+	-
Eu. Chloroplast	+	+	+	-
Eu. Nuclear	-(2)	-	-	+

(1) Eu = Eukaryotic

(2) = refers to generally less effective hybridization, see Example 4, infra.

The Table shows which probes can generally be maximally hybridized with which unknown DNA. For example, one can identify a eukaryotic organism by extracting species specific mitochondrial or chloroplast DNA, endonuclease-digesting it and hybridizing the digest with either a prokaryotic probe, or with an organelle derived eukaryotic probe. In the same manner, one can identify a prokaryotic organism by extracting species-specific cellular DNA, endonuclease-digesting it, and hybridizing the digest with either a prokaryotic probe, or an organelle-derived eukaryotic RNA probe. Also, one can identify a eukaryotic organism by extracting and digesting species-specific nuclear DNA, and hybridizing it with a non-organelle derived eukaryotic probe. Eukaryotes could be defined by one or any combination of the nuclear, mitochondria, or in some cases chloroplast systems. These cross-hybridizations are based on the fact that nucleic acid derived from eukaryotic organelles has extensive homology with evolutionarily conserved sequences from prokaryotic nucleic acid, but that the same homologies are generally not present to such extent between nuclear-derived eukaryotic DNA and prokaryotic DNA.

The choice of any pair of DNA to be digested and accompanying probe is arbitrary, and will depend on the organism being identified, i.e. it will depend on the question asked. For example, in detecting a prokaryotic

species (e.g. bacteria) present in or together with a eukaryotic cell (e.g. animal or plant) for purposes of detecting and identifying an infecting agent, one may choose a prokaryotic probe and work under conditions where organelle-derived DNA is not extracted or only minimally extracted. In this manner one assures that interference between organelle-derived DNA and prokaryotic DNA is minimal. In identifying a eukaryotic species (which is not infected with a prokaryote) with a prokaryotic probe, it is best to maximize the concentration of organelle-derived DNA, as for example by separating organelles from nuclei, and then extracting only organelle DNA. If one wishes to identify a eukaryotic organism which has been infected with a prokaryotic organism, it is best to use a non-organelle, non-prokaryotic derived probe since it will generally not hybridize well with the DNA from the prokaryote.

It is preferred to use a pair (DNA and probe) from the same kingdom, or same subkingdom, or same section, or same phylum, or same subphylum, or same class, or same subclass, or same order, or same family or same tribe or same genus. It is particularly preferred to use prokaryotic probe (e.g. bacterial probe) to hybridize with prokaryotic DNA. In this manner one could detect, quantify, and identify genera, species, and strains of prokaryotic organisms. One of the most preferred prokaryotic probes is derived from bacteria, and further,

because of the ease and availability, from E. coli. The probe from E. coli can be used to identify any organism, especially any prokaryotic organism, most preferably a strain of any bacterial species. Another particularly preferred embodiment is to use eukaryotic probe derived from a given class to identify eukaryotic organisms of the same class (e.g. mammalian probe to identify mammalian organism). Most preferred is to use probe and DNA from the same subclass and/or order and/or family of organisms, (e.g. if identifying a species of mouse, it is preferred to use mouse-derived probe).

The most sensitive and useful pair systems are those where there is less evolutionary distance or diversity between the source of the probe and the unknown DNA.

The phrase "evolutionarily conserved genetic material sequence" is used in this invention to denote genetic material, e.g DNA, sequences that show homology between at least two different species of plants, animals or micro-organisms. The homology between two conserved sequences is to be such that, if one of such DNA molecules were to be detectably labelled, sufficient hybridization or annealing would occur if both single stranded DNA molecules or fragments thereof were placed together under hybridization conditions, thereby to produce a duplex of sufficient stability to be detectable by standard methodology (i.e, radiolabelling, enzyme labelling, and the like).

In EP-A-0076123 the evolutionarily conserved

sequence exemplified was that of ribosomal RNA genes. This is still a highly preferred gene sequence. However, it has been discovered that other gene sequences exist which are sufficiently conserved across the evolutionary span to be useful.

Examples of such additional sequences are those of genes or portions thereof coding for transfer RNA, or proteins denoted as belonging to the same Superfamily, or same Family, preferably same Subfamily or even same entry in Dayhoff's "Atlas of Protein Sequence and Structure", Volume 5, Supplement 3, 1978, NBR, 1979, pages 9-24, herein incorporated by reference. A Family of proteins is one wherein any two proteins differ from each other by less than 50% amino acid residues in their sequence. A Subfamily of proteins is one wherein any two proteins differ from each other by less than 20% amino acid residues in their sequence. An "Entry" is one wherein any two proteins differ from each other by less than 5% amino acid residues in their sequence.

Specific examples of gene sequences or appropriate portions thereof which can be used are: cytochrome C related genes, cytochrome C_3 related genes, cytochrome c_1 related, cytochrome b_5 related, ferrodoxin related, rebredoxin related, flavodoxin related, alcohol dehydrogenase related, lactate dehydrogenase related, peroxidase related, adenylate kinase related, phospholipase A_2 related, tryptophan operon related,

carboxypeptidase related, subtilisin related, penicillinase related, protease inhibitor related, somatotropin related, corticotropin related, lipotropin related, glucagon related, snake venom toxin related, plant toxin related, antibacterial toxin related, immunoglogulin related gene, ribosomal other than rRNA-related genes, heme carrier genes, chromosomal protein genes, fibrous protein genes, and the like.

The conservation of some of these additional DNA sequences is not as widespread throughout the animal, plant or microbiological domains as is that of the rRNA genes. (Thus the still preferred use of rRNA). This, however, does not constitute a serious impediment to their use since it may be possible to utilize such additional sequences to identify or characterize organisms within more limited ranges or subdomains. For example it may be possible to utilize trp D gene sequences from bacteria to generate a trp D bacterial probe and then use this probe to test within the bacterial domain. In fact, it may be possible to use a trp D probe within an even narrower domain (e.g., test for the presence of Enterobacteriaceae, or of Bacillus, etc.) with a trp D probe from the same order, family or genus. Thus, while the range of applicability of some of the additional probe sequences may not be as broad as that of rRNA probes, their applicability will nevertheless be quite effective within narrower domains.

A probe containing the conserved DNA sequence information is prepared in the same manner as the preparation of rRNA information containing probe exemplified in EP-A-0076123. The probe can thus be RNA, DNA or cDNA, and the like.

The individual steps involved in the technique will be described hereinafter broadly with reference to both eukaryotic and prokaryotic cells when applicable, or specifically for each type of cell if some difference in technique exists.

The first step is extraction of the DNA from the unknown organisms. Nuclear DNA from eukaryotic cells can be selectively extracted by standard methodology well-known to the art (see for example, Drohan, W. et al, Biochem. Biophys. Acta, 521 (1978), 1-15, herein incorporated by reference). Because organelle DNA is small and circular, spooling techniques serve to separate the non-circular nuclear DNA from the circular, organelle-derived DNA. As a corollary, the non-spooled material contains the organelle-derived DNA which can separately be isolated by density gradient centrifugation. Alternatively, mitochondria (or chloroplasts) are separated from a mixture of disrupted cells; the purified mitochondrial (or chloroplast) fraction is used for the preparation of organelle-derived DNA while the purified nuclear fraction is used to prepare nuclear DNA. (See for example Bonen L. and Gray, M. W., Nucleic Acids Research, 8:319-335 (1980)).

Prokaryotic DNA extraction is also well-known in the art. Thus, for example, an unknown bacterium present in any medium, such as an industrial fermentation suspension, agar medium, plant or animal tissue or sample or the like, is treated under well-known conditions designed to extract high molecular weight DNA therefrom. For example, cells of the unknown organism can be suspended in extraction buffer, lysozyme added thereto, and the suspension incubated. Cell disruption can be further accelerated by addition of detergents, and/or by increase in temperature. Protease digestion followed by chloroform/phenol extraction and ethanol precipitation can be used to finalize the extraction of DNA. An alternative method of extraction, which is much faster than phenol/chloroform extraction, is rapid isolation of DNA using ethanol precipitation. This method is preferably used to isolate DNA directly from colonies or small, liquid cultures. The method is described in Davis, R. W. et al: "A Manual for Genetic Engineering, Advanced Bacterial Genetics," (hereinafter "Davis"), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1980, pp. 120-121, herein incorporated by reference.

The DNA (prokaryotic or eukaryotic (nuclear or non-nuclear)) is dissolved in physiological buffer for the next step. There are a variety of possible steps to be followed after isolation of the desired DNA. One of these steps is endonuclease digestion.

Digestion of extracted DNA is carried out with restriction endonuclease enzymes. Any restriction endonuclease enzyme can be used. Preferably it is not from the same organism species as that being identified, since otherwise, the DNA may remain intact. (This may, in any event, identify the organism, since the enzymes are not expected to cut DNA from the species of their origin.) Since the organism species being characterized may be unknown, obtaining a suitable digest of fragments may entail a minimum amount of trial and error, which can routinely be carried out by those skilled in the art without undue experimentation. Examples of possible restriction endonuclease enzymes are Bgl I, BamH I, EcoR I, Pst I, Hind III, Bal I, Hga I, Sal I, Xba I, Sac I, Sst I, Bcl I, Xho I, Kpn I, Pvu II, Sau IIIa, or the like. See also Davis, supra, at pp. 228-230, herein incorporated by reference. A mixture of one or more endonucleases can also be used for the digestion. Normally, DNA and endonuclease are incubated together in an appropriate buffer for an appropriate period of time (ranging from 1 to 48 hours, at temperatures ranging from 25°C-65°C, preferably 37°C).

The resulting identifying genetic characterization will depend on the type or types of endonucleases utilized, and will be endonuclease-specific. It is therefore necessary to note which enzyme or enzymes have been used for the digestion since comparative character-

izations used in a catalog should have been prepared using the same enzyme or enzymes.

An alternative step is to define endonuclease sites on the desired DNA molecules without digestion thereof, for example, by sequencing and reference to a restriction site library. Obviously, digestion is the more efficient method of noting such sites, but the method need not be limited thereto. The essence of the invention is the discovery that the position of conserved sequences along DNA, relative to the position of endonuclease restriction sites, forms a set which is characteristic for each species. Thus, any technique which yields the desired information (position of the genes vis a vis position of the sites) will be useful in the invention.

Also, the position of the conserved sequences along the DNA molecule is best noted by use of a hybridization probe. This probe is allowed to anneal to restriction fragments of the unknown's DNA. However, any other method that would allow the determination of the conserved DNA sequences, such as sequencing, would also be useful. When using the hybridization probe it is preferred to first digest and separate DNA fragments according to size, and then to hybridize the separated fragments. However, it is possible to first digest and anneal DNA with a molar excess of probe and/or sequences complementary to probe and then separate the mixture. For example, unknown DNA can be digested with a

restriction endonuclease, denatured, and hybridized in liquid with a molar excess of one or more small detectably labeled DNA fragments or synthetic oligodeoxyribonucleotides complementary to a portion or portions of the conserved sequence of interest. Since most restriction enzymes cut fairly infrequently in DNA, in most cases the double-stranded region or regions of the hybrid will be small relative to the size of the restriction fragment. The hybridization reaction is conducted under conditions where only the oligodeoxyribonucleotides hybridize. The unreacted, single-stranded DNA fragments, and the DNA fragments containing the hybridized oligodeoxyribonucleotides are separated by conventional chromatographic techniques. The labeled DNA fragments will appear in predictibly sized fractions. It is also possible to first anneal DNA with a molar excess of probe, then digest, and then separate the mixture. When the solution is incubated for a short time period or to a low C_ot, the restriction sites will be limited to the hybridized, double-stranded regions. When the solution is incubated for a long time period or to a high C_ot, the unknown DNA will anneal, thus creating a labeled duplex susceptible to restriction endonuclease cleavage. Unreassociated single-stranded tails may be removed with a nuclease such as S1. Unpaired bases may be filled in using DNA polymerase I or T4 polymerase.

Alternatively, one could find subsequences within the conserved sequence information (e.g. 20-, 30-, or 50-mers), which are more highly conserved than the remainder of the conserved region chosen as the probe. Those "shorter" sequences can be made synthetically or enzymatically, if desired, and may incorporate labeled nucleotides. Single-stranded, predigested DNA from the unknown is allowed to incubate with these shorter, highly conserved fragments and allowed to hybridize thereto. Separation would then be carried out on the digest mixture containing fragments partly annealed to the shorter labeled probes. (Thus, separation would occur after hybridization.) Separation could be by liquid chromatography, since the digest mixture would for all practical purposes behave as a mixture of essentially single-stranded fragments.

As indicated, a preferred method is to first digest, then separate, and then hybridize. Thus, after endonuclease digestion, the incubation mixture, which contains fragments of varying sizes, is preferably separated thereinto by an appropriate chromatographic method. Any method which is capable of separating nucleic acid digests according to size, and which allows the eventual hybridization with the nucleic acid probe when hybridization is the last step, can be used. For example, gel electrophoresis, high pressure liquid chromatography or capillary zone electrophoresis can be

used. (Jorgenson, J.W., J. of HRC and CC, 4: 230-231 (1981)). Presently preferred is gel electrophoresis, most preferred is agarose gel electrophoresis. In this system, the DNA digests are normally electrophoresed in an appropriate buffer, the gels are normally immersed in an ethidium bromide solution, and placed on a UV-light box to visualize standard marker fragments which may have been added. Detectably labeled standard marker fragments may be used as well.

After separation and visualization, the DNA fragments are transferred onto nitrocellulose filter paper or onto charge-modified nylon membranes by the method of Southern (Journal of Molecular Biology, 38:503-517 (1975)). The transfer can be carried out after denaturation and neutralization steps, and is usually done for long periods of time (approximately 10-20 hours) or, alternatively by means of an electrically driven transfer from gel to paper. Instruments used to accelerate the transfer from gel to paper are commercially available. The receiving nitrocellulose filter papers are then normally baked at high temperatures (60-80°C) for several hours, to bind the DNA to the filter.

Alternatively, transfer can be avoided by using the recent method of direct hybridization of Purrello, M. et al, Anal. Biochem., 128: 393-397 (1983).

The probe utilized for the hybridization of the paper-bound DNA digest fragments is a defined nucleic

acid probe preferably from or derived from a given well-defined organism or the base sequence is known.

Alternatively, the probe sequence may not have a natural conterpart; i.e., it may be a consensus sequence with a base at each position that is most commonly present at that residue in a number of equivalent sequences in different species. The consensus sequence is then generally able to form a more stable hybrid than any one of the naturally occurring sequences. The probe may be a synthetic oligodeoxyribonucleotide molecule made by covalently attaching individual nucleotides in a predetermined sequence. Synthetic molecules may be prepared, for example, by the triphosphate method of synthesis (Alvarado-Urbina et al, Science 214: 270-274 (1981)). The probe molecules may be of any useful size, and more than one sequence may be in the probe solution. For example, several 20 base sequences might be used to detect several highly conserved regions in rRNA genes. It may be detectably labeled or non-labeled, preferably detectably labeled. In such case, it is either detectably labeled RNA, but preferably nick-translated labeled DNA, cloned DNA, or detectably labeled DNA which is complementary to the RNA from the probe organism (cDNA), all of which contain highly conserved DNA sequence information. Synthetic oligodeoxyribonucleotides may be prepared with detectably labeled nucleotides, so the molecule is labeled by

incorporating labeled nucleotide residues. Depending on the choice of pair, the probe may be from a prokaryote, or from a eukaryote (cytoplasm-derived, or organelle derived). Most preferably, the detectable label is a radioactive label such as radioactive phosphorus (e.g., ^{32}P, ^{3}H or ^{14}C) or a biotin/avidin-based system. The nucleic acid probe may also be labeled with metal atoms. For example, uridine and cytidine nucleotides can form covalent mercury derivatives. Mercurated nucleoside triphosphates are good substrates for many nucleic acid polymerases, including reverse transcriptase (Dale et al, Proceedings of the National Academy of Sciences 70:2238-2242, 1973). Direct covalent mercuration of natural nucleic acids has been described. (Dale et al, Biochemistry 14:2447-2457). Reannealing properties of mercurated polymers resemble those of the corresponding nonmercurated polymers (Dale and Ward, Biochemistry 14:2458-2469). Metal labelled probes can be detected, for example, by photo-acoustic spectroscopy, x-ray spectroscopy, e.g., x-ray fluorescence, x-ray absorbance, or photon spectroscopy.

The isolation and preparation of any desired conserved DNA sequence-containing probe is within the skill of the art. For example, the isolation of rRNA from eukaryotes or prokaryotes is well-known in the art. Thus, to prepare rRNA from eukaryotic cytoplasmic ribosomes, RNA can be extracted from whole cells or

ribosomes, separated by sucrose gradient centrifugation, and the 18S and 28S fractions can be collected using known molecular weight markers. (See for example, Perry, R. P. and Kelly, D. E., "Persistent Synthesis of 5S RNA When Production of 28S and 18S Ribosomal RNA is Inhibited by Low Doses of Actinomycin D," J. Cell. Physiol., 72:235-246 (1968), herein incorporated by reference). As a corollary, organelle-derived rRNA is isolated and purified from the organelle fractions in the same manner (see e.g. Van Etten, R. A. et al, Cell, 22:157-170 (1980), or Edwards, K. et al, Nucleic Acids Research, 9:2853-2869 (1981)).

If radioactively labeled probe is used, the same is isolated from the probe organism after growth or cultivation of the organism with nutrients or in culture media containing appropriately radioactive compounds. When the probe is complementary DNA (cDNA), the same is prepared by reverse transcribing isolated RNA from the probe organism, in the presence of radioactive nucleoside triphosphates (e.g., ^{32}P-nucleosides or ^{3}H-nucleosides).

The labeled probe may also be a nick-translated DNA molecule, especially one obtained from organelle-derived whole circular DNA. In this embodiment, chloroplast or mitochondrial DNA is nick-translated in the presence of radiolabel, and a labeled DNA probe is thereby obtained. The chloroplast labeled probe will hybridize best with chloroplast DNA, and the mitochondrial labeled

probe will hybridize best with mitochondrial DNA. The chloroplast (or mitochondrial) nick-translated labeled probe will hybridize second best with mitochondrial (or chloroplast) DNA; it will also hybridize, albeit generally in less favorable fashion, with whole plant (or animal) DNA. The probe may also be obtained from eukaryotic nuclear DNA by nick-translation, although practical considerations would rule against this mode. A more useful approach in this embodiment is to cut out the highly conserved genes from the nuclear eukaryotic DNA (by restriction enzymes), separate the fragments, identify the gene sequences (as by hybridization), and isolate said gene sequences (as by electrophoresis). The isolated sequences may then be recombined into a plasmid or other vector, and after transformation of an appropriate host, cloned in ^{32}P-containing media. Alternatively, the transformed host is grown, and the DNA is then isolated and labeled by nick-translation; or the DNA is isolated, the sequences are cut out and then labeled. The resulting ribosomal probe will hybridize in the same instances as cDNA (see infra).

The preferred nucleic acid probe is radioactively labeled DNA complementary to RNA from the probe organism. The RNA is usually messenger RNA coding for a conserved gene and is substantially free of other RNA's such as transfer RNA (tRNA) or ribosomal RNA (rRNA) (unless rRNA is used). If rRNA were to be used,

prokaryotic rRNA normally contains three subspecies: the so-called 5S, 16S and 23S fragments. The reverse transcription into cDNA can be carried out with a mixture of all three, or alternatively, with a mixture of 16S and 23S fragments. It is less preferred to carry out the reverse transciption with only one of the rRNA components, although under certain conditions this may be feasible. Eukaryotic rRNA normally contains two subspecies: 18S and 28S, and the reverse transcription into cDNA can be carried out with a mixture of 18S and 28S fragments or with each.

The pure RNA, substantially free of other types of RNA, is incubated with any reverse transcriptase capable of reverse transcribing it into cDNA, preferably with reverse transcriptase from avian myeloblastosis virus (AMV) in the presence of a primer such as calf thymus DNA hydrolysate. The mixture should contain appropriate deoxynucleoside triphosphates, wherein at least one of said nucleosides is radioactively labeled, for example with ^{32}P. For example, deoxycytidine 5'-(^{32}P), deoxythymidine 5'-(^{32}P), deoxyadenine 5'-(^{32}P), or deoxyguanidine 5'-(^{32}P) triphosphates can be used as the radioactive nucleosides. After incubation, from 30 minutes to 5 hours at 25°C-40°C, extraction with chloroform and phenol, and centrifugation as well as chromatography, the radioactively labeled fractions are pooled, and constitute the cDNA probe. The radioactively labeled

cDNA probe containing conserved DNA information in substantially purified form, i.e., free of non-labeled molecules, free of cDNA which is complementary to other types of RNA, free of proteinaceous materials as well as free of cellular components such as membranes, organelles and the like, also constitutes an aspect of the present invention. A preferred probe is prokaryotic labelled cDNA, most preferred being the bacterial labelled cDNA. The probe species can be any bacterial microorganism, such as those of the family Enterobacteriaceae, Brucella, Bacillus, Pseudomonas, Lactobacillus, Haemophilus, Mycobacterium, Vibrio, Neisseria, Bactroides and other anaerobic groups, Legionella, and the like. Although the prokaryotic examples in the present application are limited to the use of E. coli as a bacterial prokaryotic probe organism, this aspect of the invention is by no means limited to this microorganism. The use of cDNA in radioactively labeled form as the probe is preferred to the use of radioactively labeled RNA because DNA has greater stability during hybridization.

It is important to recognize that the labeled cDNA probe should be a faithful copy of the RNA, i.e. be one wherein all nucleotide sequences of the template RNA are transcribed each time the synthesis is carried out. The use of a primer is essential in this respect. That the cDNA is a faithful copy can be demonstrated by the fact that it should have two properties following hybridization:

1. The cDNA should protect 100% of labeled RNA from ribonuclease digestion; and

2. The labeled cDNA should specifically anneal to the RNA as shown by resistance to S1 nuclease.

Beljanski M. M. et al, C.R. Acad. Sc Paris t 286, Serie D. p. 1825-1828 (1978), described ^3H radioactively labeled cDNA derived from E. coli rRNA. The cDNA in this work was not prepared with reverse transcriptase in the presence of a primer as in the present invention, but was prepared with a DNA polymerase I, using as a template rRNA which had been pre-cleaved using ribonuclease U_2. The rRNA digestion product (with RNAse U_2) of Beljanski et al has a different base ratio from the initial rRNA, indicating a loss of bases and/or loss of short fragments. Thus the cDNA obtained therefrom is not a faithful copy. In addition, the use of DNA polymerase I used by Beljanski is known to favor predominance of homopolymeric over heteropolymeric transcription of rRNA (see Sarin, P. S. et al, Biochem. Biophys. Res. Comm., 59:202-214 (1974)).

In sum, the probe can be seen as being derived a) from genome DNA containing conserved sequences, e.g. genes, by cloning and/or nick-translation, b) from RNA itself or c) from cDNA by reverse transcription of RNA.

Normally, the next step in the process of the invention is the hybridization of the separated DNA digest from the unknown organism with the unlabeled or

(preferably) radioactively labeled RNA or DNA probe. Hybridization is carried out by contacting the paper containing covalently labeled DNA digest from the unknown, with a hybridization mix containing the probe. Incubation is carried out at elevated temperatures (50-70°C) for long periods of time, filter papers are then washed to remove unbound radioactivity (if needed), air dried and readied for detection. An alternative, highly preferred hybridization, which is much more rapid than the one described above, is the room temperature phenol emulsion reassociation technique of Kohne, D. E. et al, Biochemistry, 16:5329-5341 (1977), which is herein incorporated by reference.

After hybridization, the technique requires selective detection of the appropriately hybridized fragments. This detection can be carried out by taking advantage of the double strandedness of the hybridized fragments and using a selective method therefor (for non-labeled probe), or by autoradiography or by an appropriate radiation scanner which may or may not be computerized, and which may increase the speed of detection (for labeled probe). These techniques are well known to those skilled in the art and will not be further described at this point.

The end product of the technique is an identifying genetic characterization, such as a chromatographic band pattern having peaks and troughs, or preferably, light

and dark regions of various intensities, at specific locations. These locations can be readily matched to specific fragments sizes (in kilobase pairs) by introduction into the separation technique of a marker, such as EcoR I digested λ bacteriophage DNA. In this manner, both the relative position of the bands to each other, as well as the absolute size of each band can be readily ascertained. The identifying genetic characterization for the unknown is then compared with characterizations present in a catalog or library. The catalog or library can consist of a book containing characterizations for at least two, and up to a virtually unlimited number of defined different organisms genera and species. For example, the number of pathologically relevant bacteria that cause human disease is estimated to be about 100, so it is estimated that a standard catalog of pathogenic bacteria would contain anywhere between 50 and 150 such characterizations. A catalog of types of bacterial strains for epidemiological typing systems can also be included.

The characterizations will depend on the type or types of endonuclease enzymes selected, possibly on the particular organism used as the source for the radioactively labeled probe (the probe organism), and on the composition of the conserved DNA sequence information nucleic acids utilized to prepare the probe (e.g. containing either prokaryotic rRNA 5S, 16S or 23S

subtypes, or only 16S and 23S, or consensus sequences or the like). Thus, the catalog may, for each probe, contain a variety of enzyme-specific characterizations, with the size of each band listed, and with the relative intensity noted. As the concentration of the bound DNA bound to the filter decreases, only the most intense bands can be seen, and the size of this band or bands can thus identify species. Any variation or permutation of the above can of course be used for the library. Additionally, for a eukaryotic organism the library may contain patterns that result from the use of one type of DNA or any combination of organelle and/or nuclear DNA. The pattern for each DNA digest will depend on the probe composition. The catalog may be arranged so that if more than one strain or species is present in the extracted sample and detected by the probe, the resulting characterization can be interpreted.

A user can either compare the obtained characterization, e.g., band pattern, visually, or by aid of a one-dimensional, computer assisted, digital scanner programmed for recognition of patterns. These computer scanners are well known in the art of the time-of-sale transactions (the commonly utilized "supermarket" check-out bar code or pattern readers). Ideally, the library or catalog is present in a computer memory both in terms of the relative characterizations for a plurality of organisms, and in terms of the absolute values of molecular weight

or size of the fragments. The catalog comparison then consists of matching the unknown characterization with one of the characterizations present in the library by means of either one or both of the stored information elements (relative characterizations and/or absolute size elements). The intensity of each band when compared to a standard can also reveal the amount of bound DNA hybridized, and thus can be used to estimate the extent of the presence of an organism, for example a prokaryote in a eukaryote.

If a user wishes to further confirm the nature and identification of a given organism, such user can digest the unknown with a second, different endonuclease, and compare the resulting characterization to catalog characterizations of the organism for the second chosen endonuclease. This process can be repeated as many times as necessary to get an accurate identification. Normally, however, a single analysis with a single probe would be sufficient in most instances.

The present invention and its variations can be used for a myriad of applications. It may be used by plant or animal breeders to correctly identify their subjects, or it may be used by clinical and microbiological laboratories to identify bacteria, parasites or fungi present in any medium, including in eukaryotic cells. In this latter use, the method is preferred to the standard microbiological assays, since it does not require

microbiological assays, since it does not require isolation and growth of the microbes. _In vitro_ growth and characterization is now either impossible for some microorganisms such as _Mycobacterium leprae_ (agent of leprosy), impossible on standard media for some microorganisms such as the obligate intracellular bacteria (e.g. rickettsia, chlamydia, etc), or highly dangerous (e.g. _B anthracis_ (agent of anthrax)). The present method depends on the isolation of nucleic acid and avoids these problems since it avoids conventional bacterial isolation and characterization. The method is expected to detect microorganisms that have not yet been conventionally described. In addition, the present method allows distinguishing different strains of species, and this can be useful, for example, for epidemiological typing in bacteriology. The method can be used by forensic laboratories to correctly and unambiguously identify plant or animal tissues in criminal investigations. It can also be used by entomologists to quickly identify insect species, when ascertaining the nature of crop infestations.

In addition, upon the conjunction of the method with the identification of infrasubspecific taxons (such as e.g., nitrogenase genes in plant roots, see Hennecke, H. 291 _Nature_ 354 (1981)), the methodology can be utilized to search for and identify the genotypes of individual strains.

The method of this invention is preferably used for the identification of microorganisms wherever they may be found. These microorganisms may be found in physiological as well as non-physiological materials. They may be found in industrial growth media, culture broths, or the like, and may be concentrated for example by centrifugation. Preferably, the microorganisms are found in physiological media, most preferably they are found in animal sources infected therewith. In this latter embodiment, the method is used to diagnose bacterial infections in animals, most preferably in humans. The detection and identification of bacterial DNA with a prokaryotic probe is highly selective and occurs without hindrance, even in the presence of animal, (e.g., mammalian) DNA. If a prokaryotic probe is used, conditions can be selected which minimize hybridization with mitochondrial DNA, or mitochondrial bands can be subtracted from the pattern. The technique can thus be used in clinical laboratories, bacterial depositories, industrial fermentation laboratories, and the like.

Of particular interest is the possibility of detecting, in addition to the species and strain identity of the infecting microorganism, the presence in the microorganism of any specific genetic sequences. For example, it is possible to detect the presence of antibiotic resistance sequences found on R factors, which are transmissible plasmids mediating drug resistance. One

can add labeled R-factor DNA or cloned labeled antibiotic resistance sequences to the hybridization mixture in order to correctly determine the antibiotic resistance of the organism, (an extra band or bands would appear), or one can rehybridize the once hybridized filter in the presence of added antibiotic resistance sequence probe or probes. Alternatively one could separate the unknown DNA into aliquots, and test the first aliquot for identification, the second for the presence of drug resistance sequences, the third for toxin genes, etc. Alternatively, one could use conserved gene information containing probe labeled with one radionuclide (e.g. ^{32}P) in a hybridization mixture with added R-factor probe labeled with a different radionuclide (e.g. ^{3}H or ^{14}C). After hybridization, the presence of R-factor DNA in the unknown DNA can be tested by scanning with two different scanners: one for species and strain identification (e.g. ^{32}P), the other for drug resistance, or the like (e.g. ^{3}H or ^{14}C). In this manner the lab can, without isolating and characterizing the microorganism, identify the genus and species, type the strain and test for drug resistance, possible toxin production or any other character or taxon below the rank of species that can be detected with a labeled nucleic acid sequence or probe, all in one experiment.

The R-factors are universal and cross species boundaries, so that identification can be carried out in

any bacterial genus or species with the same R-factor probe (see Tomkins, L. S. et al, J. Inf. Dis., 141:625-636 (1981)).

In addition, the presence of viruses or virus-related sequences in eukaryotes or prokaryotes can also be detected and identified in conjunction with the method of the invention: Any of the viruses described in "Manual of Clinical Microbiology", 3d edition, edited by Lennette, E.H., Amer. Soc. Microb., 1980, 774-778 can be identified, e.g., picornaviridae, caliciviridae, reoviridae, togaviridae, orthomyxoviridae, paramyxoviridae, rhabdoviridae, retroviridae, arenaviridae, coronaviridae, bunyaviridae, parvoviridae, papovaviridae, adenoviridae, herpesviridae, vidoviridae and poxviridae.

A) When the viral genome is integrated into host DNA (as with DNA viruses, for example members of Papovaviridae, and RNA viruses, for example, members of Retroviridae), high molecular weight DNA is extracted from the tissue and digested with restriction endonucleases. The overall procedure is the same as used for bacteria. The choice of a viral probe again depends on the question asked, and on the extent of homology between the "probe virus" and the viral related sequences to be detected. In order to have suitable sequence homology, it may be necessary that the probe and tissue sequences are related to the same family, genus, or species of

virus. In addition to the extent of conserved sequences, whether or not a viral probe hybridizes to viral related sequences in host DNA may be determined by the hybridization conditions, which can be stringent or relaxed. The result of the hybridization will be a band or a pattern of bands showing that there are viral sequences incorporated into the host DNA. This information may be useful in helping to predict the occurrence of cancer. The probe can be any labelled complementary nucleic acid probe including cloned viral sequences. For RNA viruses, for example viral RNA can be used to make a DNA with reverse transcriptase; for DNA viruses, for example, viral DNA labelled by nick translation can be used. Again multiple probes can be used, especially with different labels.

Same general features apply equally to DNA and RNA viruses. Viral genomes are relatively small, so the precipitated nucleic acid is preferably collected by centrifugation; all of the procedures can use the total nucleic acid or the various procedures can be run separately. It is expected that viral nucleic acid can be concentrated by spooling cellular DNA to remove it before centrifugation. This can also be used to determine if the viral genome is integrated.

For the viral probe to hybridize, it may be necessary and at least most preferred that the probe be from the same family, genus, or species as the unknown.

Reaction conditions, stringent or relaxed, may determine whether or not a given probe hybridizes a distantly related genome. The probe may be cloned viral sequences that are labeled, or may be the complete genome or a portion of it.

The technique described by Southern, supra is useful for the transfer of large DNA fragments (greater than about 0.5 kilobases) to nitrocellulose paper after alkali denaturation. This technique might be useful for DNA viruses but not for RNA viruses. RNA has been transferred and covalently coupled to activated cellulose paper (diazobenzyloxymethyl-paper), and this can be used for RNA viruses. The modification of the Southern technique by Thomas (Thomas, P., Proc. Nat. Acad. Sci., USA, 77:5201-5205 (1980)) can be used for the efficient transfer of RNA, and small DNA fragments to nitrocellulose paper for hybridization. RNA and small DNA fragments are denatured with glyoxal and dimethyl sulfoxide, and electrophoresed in agarose gel. This procedure transfers DNA fragments between 100 and 2000 nucleotides and RNA efficiently, and they are retained on the nitrocellulose paper during hybridization. This is useful for small ribosomal DNA fragments as well. So it is most preferred to divide the restriction-enzyme digested specimen and denature the nucleic acid in one portion with glyoxal. The Southern and Thomas procedures would yield a maximum amount of information.

B) For DNA viruses, restriction analysis can be carried out with double-stranded (DS) viral DNA's to identify viruses present. Single-stranded (SS) DNA viruses will have different genome lengths. The probe (the sequence information could be converted to DS DNA) that hybridizes, the hybridized fragment pattern and/or the sizes or size can be used to identify viruses. There are again a number of ways to obtain complementary nucleic acid probes. For example, for DS DNA nick-translation can be used; for SS DNA, DNA polymerase can be used to synthesize a cDNA.

C) For RNA viruses, RNA is not digested by restriction endonucleases (the sequence information could be converted to DS DNA). The genomes of different RNA viruses are of different sizes, and some RNA viruses have more than 1 molecule in their genome. This, along with the base sequences detected by certain probes or pooled probes allows the RNA viruses to be identified. An example of a probe would be cDNA synthesized using viral RNA.

When searching for infectious agents in specimens it is possible to search directly by extracting nucleic acid from the specimen, or by culturing first in media or cells to increase the number of agents, or by using a concentration step such as centrifugation, or by trying all approaches.

The present invention lends itself readily to the preparation of "kits" containing the elements necessary to carry out the process. Such a kit may comprise a carrier being compartmentalized to receive in close confinement therein one or more container means, such as tubes or vials. One of said container means may contain unlabeled or detectably labeled nucleic acid probe, such as for example the radioactively labeled cDNA to RNA from the organism probe, (preferably prokaryotic cDNA in the case of a kit to identify bacteria). The labeled nucleic acid probe may be present in lyophilized form, or in an appropriate buffer as necessary. One or more container means may contain one or more endonuclease enzymes to be utilized in digesting the DNA from the unknown organism. These enzymes may be present by themselves or in admixtures, in lyophilized form or in appropriate buffers. Ideally, the enzymes utilized in the kit are those for which corresponding catalogs have been prepared. Nothing stops the user, however, from preparing his or her own comparative standard at the moment of experiment. Thus, if a user suspects that an unknown is in fact of a given genus or species, he or she may prepare the identifying characteristics for the known and compare it with the characterization for the unknown. The kit may thus also contain all of the elements necessary in order to carry out this sub-process. These elements may include one or more known organisms, (such

as bacteria), or isolated DNA from known organisms. In addition, the kit may also contain a "catalog", defined broadly as a booklet, or book, or pamphlet, or computer tape or disk, or computer access number, or the like, having the identifying characterizations for a variety of organisms of a certain group, such as plant species, mammal species, microbe species, especially pathologically important bacteria, insect species or the like. In this mode, a user would only need to prepare the characterization for the unknown organism, and then visually (or by computer) compare the obtained characterization with the characterizations in the catalog. The kit may also contain in one container probe RNA for probe synthesis, in another container radiolabeled deoxyribonucleoside triphosphate, and in another container primer. In this manner the user can prepare his or her own probe cDNA.

Finally, the kit may contain all of the additional elements necessary to carry out the technique of the invention, such as buffers, growth media, enzymes, pipettes, plates, nucleic acids, nucleoside triphosphates, filter paper, gel materials, transfer materials, autoradiography supplies, and the like. It may also contain antibiotic resistance sequence probes, viral probes, or other specific character probes.

Having now generally described this invention, the same will be better understood by reference to certain

reference experiments and specific examples which are included herein for purposes of illustration only and are not intended to be limiting of the invention, unless specified.

MATERIALS AND METHODS

A. Bacterial

Extraction of High Molecular Weight DNA

Bacterial broth cultures were centrifuged and the cells were washed with cold saline. The cells were suspended in a volume measured in ml of extraction buffer (0.15 M sodium chloride, 0.1 M EDTA, 0.03 M tris pH 8.5) approximately 10 times the gram weight of the packed cells. Lysozyme at 10 mg/ml was added to 0.5 mg/ml final concentration. The suspension was incubated at 37°C for 30 minutes. Cell disruption was completed by the addition of 25% SDS to 2.5% final concentration, and raising the temperature to 60°C for 10 minutes. After cooling in a tap water bath, mercaptoethanol was added to 1% final concentration. Pronase® at 20 mg/ml in 0.02 M tris pH 7.4 was predigested at 37°C for 2 hours and then added to 1 mg/ml final concentration. The solution was incubated at 37°C for 18 hours. Phenol was prepared by mixing one liter redistilled phenol, 2.5 liters double distilled water, 270 ml saturated Tris base, 12 ml mercaptoethanol, and EDTA to 10^{-3}M final concentration and allowing the mixture to separate at 4°C. The phenol was washed with wash buffer (10^{-1}M sodium chloride, 10^{-3}M EDTA, 10mM tris pH 8.5). Then an equal volume of fresh buffer was added. Mercaptoethanol was added to 0.1% final concentration. The solution was mixed and stored at 4°C. One half volume prepared phenol and one half

volume chloroform was added to the lysed cell solution. This was shaken for approximately 10 minutes and centrifuged at 3,400 x g for 15 minutes. The aqueous phase was removed with an inverted 25 ml glass pipette. The extraction procedure was repeated until there was little precipitate at the interface. One-ninth volume 2 N sodium acetate pH 5.5 was added to the aqueous phase. Two times volume of 95% ethyl alcohol at -20°C was poured slowly down the side of the flask. The end of a Pasteur pipette was melted close and used to spool the precipitated DNA. High molecular weight DNA was dissolved in buffer (10^{-3}EDTA, 10^{-2}M tris pH 7.4). The concentration of DNA was determined by absorbance at 260 nm using 30 micrograms per absorbance unit as conversion factor.

Restriction Endonuclease Digestion of DNA.

EcoR I restriction endonuclease reactions were performed in 0.1 M tris-HCl pH 7.5, 0.05 M NaCl, 0.005 M $MgCl_2$, and 100 micrograms per ml bovine serum albumin. EcoR I reaction mixtures contained 5 units of enzyme per microgram of DNA, and were incubated four hours at 37°C. PST I restriction endonuclease reactions were performed in 0.006 M tris-HCl pH 7.4, 0.05 M sodium chloride, 0.006 M magnesium chloride, 0.006 M 2-mercaptoethanol, and 100 micrograms per ml of bovine serum albumin. PST I reaction mixtures contained 2 units of enzyme per microgram DNA, and were incubated four

hours at 37°C. Usually 10 micrograms DNA was digested in a final volume of 40 microliters. Ten times concentration buffers were added. Sterile distilled water was added depending on the volume of DNA. λ Bacteriophage DNA was restricted with EcoR I to provide marker bands for fragment size determinations. Usually 2 micrograms λ DNA was digested with 20 units EcoR I in a final volume of 20 microliters.

Gel Electrophoresis and DNA Transfer.

DNA digests were supplemented with glycerol, to about 20%, and bromophenol blue tracking dye. In the case of λ DNA digests, 20 microliters of 1x EcoR I buffer was added to each 20 microliter reaction mixture. Usually 15 microliters 75% glycerol and 5 microliters 0.5% bromophenol blue were added to each 40 microliter reaction mixture.

10 micrograms digested bacterial DNA or 2 micrograms digested λ DNA were loaded per well and overlaid with molten agarose. Digests were electrophoresed in 0.8% agarose with 0.02 M sodium acetate, 0.002 M EDTA, 0.018 M tris base, and 0.028 M tris HCl pH 8.05 at 35 V until the dye migrated 13 to 16 cm. Gels were then immersed in ethidium bromide (0.005 mg/ml) and placed on a UV-light box to visualize the λ fragments. DNA was transferred to nitrocellulose filter paper by the method of Southern, *supra*. Gels were treated with denaturing solution (1.5 M

sodium chloride, 0.5 M sodium hydroxide) on a rocker table for 20 min. Denaturing solution was replaced with neutralization solution (3.0 M sodium chloride, 0.5 M tris HCl, pH 7.5), and after 40 minutes the gels were checked with pH paper. Following neutralization, the gels were treated with 6 x SSC buffer (SSC = 0.15 M sodium chloride, 0.015 M sodium citrate) for 10 minutes. DNA fragments were transferred from the gel to the nitrocellulose paper by drawing 6 X SSC through the gel and nitrocellulose paper with a stack of paper towels for 15 hours. Filters were placed between two sheets of 3 MM chromatography paper, wrapped in aluminum foil, shiny side out, and dried in a vacuum oven at 80°C for 4 hours.

Synthesis of ^{32}P ribosomal RNA Complementary DNA (^{32}P rRNA cDNA).

^{32}P-labeled DNA complementary to E. coli R-13 23S and 16S ribosomal RNA was synthesized using reverse transcriptase from avian myeloblastosis virus (AMV). The reaction mixture contained 5 microliters 0.2 M dithiothreithol, 25 microliters 1 M tris pH 8.0, 8.3 microliters 3 M potassium chloride, 40 microliters 0.1 M magnesium chloride, 70 micrograms actinomycin, 14 microliters 0.04 M dATP, 14 microliters 0.04 M dGDP, 14 microliters 0.04 M dTTP and 96.7 microliters H_2O. The following were added to a plastic tube: 137.5 microliters

reaction mixture, 15 microliters calf thymus primer (10 mg/ml), 7 microliters H 20, 3 microliters rRNA (using 40 micrograms /OD unit concentration, is 2.76 micrograms/microliters), 40 microliters deoxycitydine 5'-(^{32}P) triphosphate (10 mCi/ml), and 13 microliters AMV polymerase (6,900 units μl. The enzymatic reaction was incubated 1.5 hours at 37°C. Then the solution was extracted in 5 ml each of chloroform and prepared phenol. After centrifugation (JS 13,600 RPM), the aqueous phase was layered directly on a Sephadex® G-50 column (1.5 x 22 cm). A plastic 10 ml pipette was used for the column. A small glass bead was placed in the tip, rubber tubing with a pinch clamp was attached, and degassed G-50 swelled in 0.05% SDS overnight was added. The aqueous phase wall allowed to flow directly into the G-50 and was then eluted with 0.05% SDS. 20 fractions at 0.5 ml each were collected in plastic vials. Tubes containing peak fractions were detected by Cerenkov counting using a ^{3}H discriminator, counting for 0.1 min. per sample and recording total counts. Peak fractions were pooled. Aliquots were added to Aquesol® (commercially available), and the CPM of ^{32}P per ml was determined by scintillation counting.

Hybridization and Autoradiography.

Fragments containing ribosomal RNA gene sequences were detected by autoradiography after hybridization of the DNA on the filters to ^{32}P-rRNA cDNA. Filters were soaked in hybridization mix (3 x SSC, 0.1% SDS, 100 micrograms/ml denatured and sonicated canine DNA, and Deinhart's solution (0.2% each of bovine serum albumen, Ficoll, and polyvinyl pyrrolidine)), for 1 hour at 68°C. ^{32}P rRNA cDNA was added at 4 x 10^6 CPM/ml, and the hybridization reaction was incubated at 68°C for 48 hours. Filters were then washed in 3 x SSC, and 0.1% SDS at 15 min. intervals for 2 hours or until the wash solution contained about 3,000 cpm ^{32}P per ml. Filters were air dried, wrapped in plastic wrap and autoradiographed approximately 1 hour with Kodak X-OMAT R film at -70°C.

B. Mammalian experiments. *Mus musculus domesticus* (mouse) rRNA probes were synthesized from 18S and 28S, and only 28S rRNA. Nucleic acid was extracted from mouse liver and precipitated. High molecular weight DNA was spooled and removed. The remaining nucleic acid was collected by centrifugation and dissolved in buffer, 50 mM $MgCl_2$ and 100 mM Tris pH 7.4. DNAse (RNAse free) was added to a concentration of 50μg/ml. The mixture was incubated at 37°C for 30 min. The resulting RNA was rextracted, ethanol precipitated, and dissolved in 1mM sodium phosphate buffer pH 6.8 A 5 to 20% sucrose gradient in 0.1M Tris pH 7.4 and 0.01M EDTA was pre-

pared. The sample was added and the gradients spun in an SW40 rotor 7 hr. at 35K RPM. Fractions were collected by optical density. The 18S and 28S fractions were selected by comparison to known molecular weight markers.

For all of the mammalian experiments relaxed hybridization conditions were used, 54°C. The washing procedure, carried out at 54°C, was 3 separate washes with 3xSSC with 0.05% SDS for 15 min. each.

Reference Experiments 1-8 describe experiments carried out with rRNA-information containing probes. Examples 1 to 3 describe computer simulations utilizing a histone gene information-containing probe, a tryptophan operon trp D gene-information containing probe, and an α-fetoprotein gene information-containing probe, respectively.

Reference Experiment 1

Bacterial Species are Defined by Restriction Endonuclease Analysis of Ribosomal RNA Genes

The several strains of *P. aeruginosa* used in this example have the minimal phenotypic characters which identify the species (Hugh R.H., et al, in: Manual of Clinical Microbiology, 2d Ed. ASM, 1974, pp. 250-269). (Table 2). Strains of three other *Pseudomonas* and two *Acinetobacter* species were selected to compare species and genera (Table 3).

TABLE 2

Corresponding strain numbers of isolates with the minimal phenotypic characters of P. aeruginosa for the comparison of Strains.

RH	ATCC
151	10752
809	7701
810	8689
811	8707
812	8709
815	10145
1559	14425

Strains used for comparison of Pseudomonas and Acinetobacter species are listed in Table 3.

TABLE 3

Corresponding strain numbers of type, neotype and reference strains for the comparison of species and genera

Species	RH	ATCC	NCTC	Strain Status
P. aeruginosa	815	10145	10332	type
P. stutzeri	2601	17588		neotype
P. fluorescens	818	13525	10038	neotype
P. putida	827	12633		neotype
A. anitratus	2208	19606		type
A. lwoffii	462	7976		reference

Acinetobacter species were selected for comparison of genera because they share certain attributes with many *Pseudomonas* species.

The sizes (kilobase pairs) of fragments in EcoR I digests are: *P. stutzeri* 16.0, 12.0, 9.4; *P. fluorescens* 16.0, 10.0, 8.6, 7.8, 7.0; *P. putida* 24.0, 15.0, 10.0, 8.9; *A. anitratus* 20.0, 15.0, 12.5, 9.8, 7.8, 6.1, 5.2, 4.8, 3.8, 2.8 (size of the smallest 3 fragments not calculated); *A. lwoffii* 12.0, 10.0, 9.1, 7.0, 6.4, 5.7, 5.5, 5.3, 4.8, 4.4, 3.6, 3.2, 2.9 (size of the smallest 3 fragments not calculated). The sizes (kilobase pairs) of fragments in PST I digests are; *P. stutzeri* 6.7, 6.1, 5.5; *P. fluorescens* 10.0, 9.4, 7.8, 7.0; *P. putida* 10.5,

9.9, 6.8, 6.3, 4.4; A. anitratus 36.0, 28.0, 20.5, 12.0, 10.0, 5.8, 3.7, 2.6, 2.4; A. lwoffi 9.9, 8.7, 7.2, 5.7, 4.0, 3.6, 3.2, 2.7.

Comparison of the hybridized restriction fragments from the seven strains of P. aeruginosa leads to the conclusion that this species can be defined by an EcoR I specific set of fragments containing rRNA gene sequences, 10.1, 9.4, 7.6, and 5.9 kilobase pairs (KBP) (FIGURE 1). The 7.6 KBP EcoR I fragment occurs in 4 of the 7 strains in this sample. An analogous situation occurs among certain phenotypic characters of strains of species. The fact that the EcoR I sets of fragments from the 7 strains can be used to separate the strains into two groups prompts speculation that there may be two species with the minimal phenotypic characters of P. aeruginosa. The results of experiments in which DNA was digested with PST I (FIGURE 2) lead to the conclusion that the strain variation shown by the EcoR I 7.6 KBP fragment represents variation within the species, since there is a single conserved set of PST I fragments, 9.4, 7.1, 6.6, and 6.4 KBP, that define the species. The 9.4 and 6.6 KBP Pst I fragments occur in 6 of the 7 strains of P. aeruginosa; the 7.1 and 6.4 KBP PST I fragments occur in all of the strains sampled. PST I fragment variation occurs in strains that do not contain an EcoR I 7.6 KBP fragment; RH 151 has 10.1 and 8.2 KBP fragments, RH 809 does not contain a 9.4 KBP fragment and has a 6.0

KBP fragment, and RH 815; the type strain, does not contain a 6.6 KBP fragment. The patterns of hybridized fragments support the conclusion that enzyme specific, conserved sets can be used to define species. Strains of a species probably have a majority of the fragments in the conserved set. The occurrence of fragment variations in some strains does not prevent identification and may prove useful in epidemiological studies.

The occurrence of variation, EcoR I 7.6 KBP fragment in *P. aeruginosa* strains, may be put into perspective by examining hybridized EcoR I fragments found in the type strains of other *Pseudomonas* species (FIGURE 3). The type strains of *P. stutzeri*, *P. fluorescens*, and *P. putida* do not contain a 7.6 KBP fragment, but do have EcoR I fragments of the sane size in common; *P. aeruginosa* and *P. stutzeri* each have a 9.4 KBP fragment, *P. stutzeri* and *P. fluorescens* each have a 16 KBP fragment, and *P. fluorescens* and *P. putida* each have a 10 KBP fragment. In general, the sizes of the fragments are unique in the type strains of each of the 4 *Pseudomonas* species; and the type strain of each species has a different size range of fragments. These general comments are also true for the PST I digests (FIGURE 4).

When the fragment patterns or one strain of each of the 4 *Pseudomonas* and 2 *Acinetobacter* species are compared, it can be concluded that the species of each genus are similar, but the genera differ. The 2 *Acinetobacter*

species have a greater range of hybridized fragment sizes than do the 4 Pseudomonas species.

Without the aid of restriction enzyme maps such as those availble for E. coli. Bacillus thuringiensis and B. subtilis, it is not possible to predict where enzymes cut rRNA genes, the number of copies per genome, whether there are heterologous flanking regions between genes or gene heterogeneity. The E. coli rRNA cDNA probe may fail to hybridize with some restriction fragments containing rRNA gene sequences, and if so, this reflects the evolutionary distance or diversity between the test organism and E. coli. The conserved nature of rRNA can be used to argue that this is not the case. However, this is a minor problem compared to the advantage of having a standard probe that can be equally applied to any unknown species.

Reference Experiment 2

Comparison of Restriction Analysis with DNA-DNA Liquid Hybridization:

The strains used in this study are listed in Tables 4 and 5.

Table 4

Corresponding Strain Numbers
of Neotype strains of B. subtilis
and type strains of junior synonyms

Species	RH	ATCC	Strain Status
B. subtilis	3021	6051	neotype
B. uniflagellatus	2990	15134	type
B. amyloliquafaciens	3061	23350	type

Table 5. Corresponding strain number of strains of *B. subtilis*

RH	NRRL	ATCC
3063	B-354(NRS-231)	6633
3064	B-356(NRS-238)	7067
3065	NRS-265	6455
3066	NRS-659	7060
3067	NRS-730	7003
3068	NRS-737	943
3069	NRS-741	4344
3070	NRS-773	8188
3071	NRS-1106	4944
3072	NRS-1107	7480

High molecular weight DNA was isolated from each of the strains. Liquid DNA-DNA hybridization data was collected using RH 3021 and RH 2990 labeled DNAs and results are shown in Table 6.

TABLE 6

Percent hybridization between labeled DNA probe and DNA from strains of *B. subtilis*

Labeled DNA probe	RH 3063	RH 3064	RH 3066	RH 3067	RH 3068	RH 3065
RH 3021	61	77	51	96	84	18
RH 2990	12	10	13	15	16	50

	RH 3069	RH 3070	RH 3071	RH 3072	RH 3021	RH 2990
RH 3021	14	—	93	15	100	20
RH 2990	100	—	17	100	20	100

	RH 3061
RH 3021	11
RH 2990	70

The data shows there are two hybridization groups. Similar data is reported in the literature for _B. subtilis_ (_Seki et al_, _International Journal of Systematic Bacteriology_ _25_:258-270 (1975)). These two groups can be represented by RH 3021 and RH 2990. When restriction endonuclease analysis of ribosomal RNA genes is carried out, EcoR I digests (FIGURE 5) can be separated into two groups. The group represented by RH 3021 has two intensely hybridized fragments (2.15 and 2.1 KBP). The group represented by RH 2990 has two intensely hybridized fragments (2.6 and 2.5 KBP). The EcoR I data can be used to place _B. subtilis_ strains in appropriate DNA-DNA hybridization groups. According to the DNA-DNA hybridization 70% rule, _B. subtilis_ is actually two species. However, when the PST I data (FIGURE 6) is considered, it is possible to think of the groups as two divergent populations related to a common ancestor or speciation event. The conclusion that _B. subtilis_ is one species correlates with phenotypic data. The strains listed in Table 5 are identified as _B. subtilis_ in _Gordon, R. E. et al_ "The Genus Bacillus", _Agriculture Handbook No. 427_, Agricultural Research Service, U.S. Dept. of Agriculture, Washington, D.C. pp. 36-41. Restriction analysis can provide data which is comparable to DNA-DNA hybridization data, or by selecting the proper enzyme, restrction analysis can adequately define species despite divergence. RH 3061 has lost PST I sites. How-

ever, the EcoR I data suggests that the strain is B. subtilis. The same is concluded from the Bgl II data (FIGURE 7) and Sac I data (FIGURE 8).

Reference Experiment 3

Stability of the Restriction Analysis Pattern and Other Bacillus polymyxa Experiments

TABLE 7

Neotype strains of B. subtilis and B. polymyxa

Species	RH	ACTT	NRRL	Comments
B. subtilis	3021	6051		neotype
B. polymyxa	3074	842		neotype
B. polymyxa	3033			asporogenous mutant derived from RH 3074
B. polymyxa	3062		NRS-1105	neotype
B. polymyxa	3073			asporogenous mutant derived from NRS-1105

B. subtilis and *B. polymyxa* can be distinguished by EcoR I data (FIGURE 9), PST I data (FIGURE 10) Bgl II data (FIGURE 11, left) and Sac I data (FIGURE 11, right). It can be concluded from the major differences in the PST I band patterns that *bacillus polymyxa* is in the wrong genus. While both species produce spores, they are not phenotypically similar. It is reassuring that the type strain of *B. polymyxa* from both culture collections, ATCC and NRRL have the same band patterns. The important data, however, is that the asporogenous mutants can be identified. It is very difficult, perhaps impossible, to identify *Bacillus* species if they fail to form spores.

Reference Experiment 4

Identification of a Bacterial Species in Mouse Tissue without Isolation

A Swiss mouse, *Mus musculus domesticus* (inbred strain), was inoculated intraperitoneally with 0.5 ml of a turbid suspension of *Streptococcus pneumoniae* RH 3077 (ATCC 6303). When the mouse became moribund, the heart, lungs, and liver were removed. High molecular weight DNA was isolated from these tissues, *S. pneumoniae* RH 3077 and Swiss mouse organs, and the procedure for restriction endonuclease analysis of rRNA genes was carried out using EcoR I to digest the DNAs. In addition to washing the filters in 3 X SSC, they were washed for 2 X 15 minute periods in 0.3 X SSC and 0.05% SDS. Autoradiography was

carried out for 48 hr. The data (FIGURE 12) shows that S. pneumoniae can be defined by seven hybridized fragments (17.0, 8.0, 6.0, 4.0, 3.3, 2.6 and 1.8 KBP). The bacterial cDNA probe hybridizes poorly to two mouse DNA fragments (14.0 and 6.8 KBP). Hybridized fragments signal the presence of S. pneumoniae in the infected tissues. All seven bands can be seen in the heart DNA extract. They are less intense in the liver DNA extract, but all can be seen in the autoradiograph. Only the 6.0 KBP band appears in the lung DNA extract. The lesser number of bacteria in the lungs can be explained by the mouse having septicemia rather then pneumonia. The lungs showed no consolidation at autopsy. In order to determine the sensitivity of the assay, bacterial DNA was diluted with mouse DNA and electrophoresed. All seven bands can be seen in the autoradiograph when 0.1 micrograms of bacterial DNA is used. The 17.0, 8.0 and 6.0 KBP bands can be seen with 10^{-3} μg of bacterial DNA. If the figure of 5 X 10^{-3} μg DNA per 10^{6} S. pneumoniae cells is used (Biochim Biophys Acta, 26:68), 10^{-1} μg is equivalent to 2 X 10^{7} cells. The present technique is thus useful for diagnosing infections at this level of sensitivity.

This reference experiment also demonstrates that the bacterial probe hybridizes with mouse-specific EcoR I fragments (see FIGURE-9, fragments having 14.0 and 6.8 KBP). These gragments correspond to EcoR I fragments detected by

mouse 18S and 28S ribosomal RNA probe (FIGURE 14 _infra_ shows that the 6.8 KBP fragment contains the 28S rRNA sequences). The bacterial probe does not hybridize as well to mammalian ribosomal RNA gene sequences, so the bands are less intense, the system of bacterial probe and nuclear mammalian DNA is less sensitive, and selectivity for DNA from infecting prokaryotes is clearly demonstrated. In experiments where bacterial probe was hybridized to 10 μg digested bacterial DNA per lane, no hybridization to 10 μg digested human or mouse DNA per lane was detected on the autoradiographs when the bacterial bands were clearly seen.

Reference Experiments 5-8

Mammalian experiments

These reference experiments illustrate that the concept of rRNA restriction analysis to identify organisms can be successfully applied not only to bacteria but to complex, eukaryotic organisms.

FIGURE 13 shows that mammelian genera can be recognized with _Mus musculus domesticus_ 18S and 28S rRNA probe, and that several species of _Mus_ can be distinguished. In this figure, the enzyme is PST I and the subjects and corresponding bands are as follows:

1. _Mus musculus melossinus_ (mouse) 14.5, 13.5, 2.6
2. _Mus musculus domesticus_ (mouse) 13.5, 2.6
3. _Canis familiaris_ (dog) 12.0

4. *Cavia porcellus* (guinea pig) 17.0, 14.0, 13.0, 8.8, 5.7, 4.7, and one band less than 3.0

5. *Cricetulus griseus* (hamster) 25.0, 4.7

6. *Homo sapiens* (human) 15.0, 5.7

7. *Felis catus* (cat) 20.0, 9.7

8. *Ratus norvegicus* (rat) 12.5

9. *Mus musculus domesticus* (mouse) 13.5, 2.6

10. *Mus cervicolor cervicolor* (mouse) 14.0, 2.7

11. *Mus cervicolor papeus* (mouse) 13.5, 2.6

12. *Mus pahari* (mouse) 13.0, 3.7

13. *Mus cookii* (mouse) 13.5, 2.6

FIGURE 14 shows that mouse and cat DNA can be distinguished by the 28S rRNA cDNA alone, and that the pattern of hybridized bands is dependent on the composition of the probe sequences. In Figure 14 the enzyme is EcoR I, and the subjects and bands are as follows:

1. *Mus musculus domesticus* (mouse) 6.8 KBP
2. *Felis catus* (cat) 8.3 KBP

In Figure 15 the enzyme is Sac I, and the subjects and bands are as follows:

1. *Erythrocebus patas* (patas monkey) 8.5, 3.7, <3.0
2. *Ratus norvegicus* (rat) 25.0, 9,5, 3.6, <3.0
3. *Mus musculus domesticus* (mouse) 6.8, <3.0
4. *Felis catus* (cat) 9.5, 5.3, 4.0, <3.0, <3.0
5. *Homo sapiens* (human) 10.5, <3.0

6. *Macaca mulatta* (rhesus monkey) 9.8, <3.0 When Figure 15 (Sac I digests) is compared to the other mammalien figures it can be seen the the hybridized pattern is enzyme specific.

Figure 16 shows that primates can be distinguished. Cell cultures have bands in common with tissue from the species of origin, and different human cell cultures can be distinguished by additions and deletions of bands. In this figure, the enzyme is EcoR I, and the subjects and bands are as follows:

1. *Erythrocebus patas* (patas monkey) >22.0, 11.0, 7.6, 2.6

2. *Macaca mulatta* (rhesus monkey) 22.0, 11.5, 7.6

3. *Homo sapiens* (human) >22.0, 22.0, 16.0, 8.1, 6.6

4. M 241/88 (langur monkey cell culture) 14.0, 7.2, 5.7

5. HeLa (human cell culture) >8,1, 6.6

6. J96(human cell culture) > 22.0, 22.0, 16.0, 11.0, 8.1, 6.6

7. AO (human cell culture) 22.0, 16.0, 8.1, 6.6

8. X-381 (rhesus monkey) 22.0, 11.5, 7.6

Example 1

Use of an H4 Histone Gene Probe

A computer simulation of the identification and characterization of two animal species (sea urchin and mouse) was carried out using a conserved DNA sequence derived from the H4 histone gene.

The histone H4 gene sequence for sea urchin (Psammechinus miliaris) is shown below, where A, T, C, G, represent the known nucleotides, and N represent a presently unknown position (788 base pairs).

```
        10         20         30         40         50
CAACATATTA GAGGAAGGGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA
GTTGTATAAT CTCCTTCCCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT

        60         70         80         90        100
GGGGGGGGGG GAGGGAGAAT TGCCCAAAAC ACTGTAAATG TAGCGTTAAT
CCCCCCCCCC CTCCCTCTTA ACGGGTTTTG TGACATTTAC ATCGCAATTA

       110        120        130        140        150
GAACTTTTCA TCTCATCGAC TGCGCGTGTA TAAGGATGAT TATAAGCTTT
CTTGAAAAGT AGAGTAGCTG ACGCGCACAT ATTCCTACTA ATATTCGAAA

       160        170        180        190        200
TTTTCAATTT ACAGGCACTA CGTTACATTC AAATCCAATC AATCATTTGA
AAAAGTTAAA TGTCCGTGAT GCAATGTAAG TTTAGGTTAG TTAGTAAACT

       210        220        230        240        250
ATCACCGTCG CAAAAGGCAG ATGTAAACTG TCAAGTTGTC AGATTGTGTG
TAGTGGCAGC GTTTTCCGTC TACATTTGAC AGTTCAACAG TCTAACACAC

       260        270        280        290        300
CGCGGCCTCC AGTGAGCTAC CCACCGGGCC GTCGCGGAGG GGCGCACCTG
GCGCCGGAGG TCACTCGATG GGTGGCCCGG CAGCGCCTCC CCGCGTGGAC

       310        320        330        340        350
TGCGGGAGGG GTCATCGGAG GGCGATCGAG CCTCGTCATC CAAGTCCGCA
ACGCCCTCCC CAGTAGCCTC CCGCTAGCTC GGAGCAGTAG GTTCAGGCGT

       360        370        380        390        400
TACGGGTGAC AATACCCCCG CTCACCGGGA GGGTTGGTCA ATCGCTCAGC
ATGCCCACTG TTATGGGGGC GAGTGGCCCT CCCAACCAGT TAGCGAGTCG
```

```
       410        420        430        440        450
GAAACGTCCA GTCGTCAGCA TCGCACTAAG ACTCTCTCTC AATCTCCATA
CTTTGCAGGT CAGCAGTCGT AGCGTGATTC TGAGAGAGAG TTAGAGGTAT

       460        470        480        490        500
ATGTCAGGCC GTGGTAAAGG AGGCAAGGGG CTCGGAAAGG GAGGCGCCAA
TACAGTCCGG CACCATTTCC TCCGTTCCCC GAGCCTTTCC CTCCGCGGTT

       510        520        530        540        550
GCGTCATCGC AAGGTCCTAC GAGACAACAT CCAGGGCATC ACCAAGCCTG
CGCAGTAGCG TTCCAGGATG CTCTGTTGTA GGTCCCGTAG TGGTTCGGAC

       560        570        580        590        600
CAATCCGCCG ACTCNNNNNN NNNNNNNNNN NNNNNNGAAT CTCTGGTCTT
GTTAGGCGGC TGAGNNNNNN NNNNNNNNNN NNNNNNCTTA GAGACCAGAA

       610        620        630        640        650
ATCTACGAGG AGACACGAGG GGTGCTGAAG GNNNNNNNNN NNNNNNNNNN
TAGATGCTCC TCTGTGCTCC CCACGACTTC CNNNNNNNNN NNNNNNNNNN

       660        670        680        690        700
NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN

       710        720        730        740        750
NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNGGCCGAAC ACTGTACGGC
NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNCCGGCTTG TGACATGCCG

       760        770        780
TTCGGCGGCT AAGTGAAGCA GACTTGGCTA GAATAACG
AAGCCGCCGA TTCACTTCGT CTGAACCGAT CTTATTGC
```

The analogous mouse H4 gene sequence is shown below (968 base pairs):

```
        10         20         30         40         50
GAATTCTCCG AGGGACTTCG GCACCATAAT TAAGAAAATC GAAAATAAAA
CTTAAGAGGC TCCCTGAAGC CGTGGTATTA ATTCTTTTAG CTTTTATTTT

        60         70         80         90        100
AAATAAAGGC TTGAGACTGT AAGGAACCGG TAGAGGGCAG AGAAGAGAAA
TTTATTTCCG AACTCTGACA TTCCTTGGCC ATCTCCCGTC TCTTCTCTTT

       110        120        130        140        150
AGAAAAACAG GAAGATGATG CAACATCCAG AGCCCGGATA ATTTAGAAAG
TCTTTTTGTC CTTCTACTAC GTTGTAGGTC TCGGGCCTAT TAAATCTTTC

       160        170        180        190        200
GTTCCCGCCC GCGCGCTTTC AGTTTTCAAT CTGGTCCGAT CCTCTCATAT
CAAGGGCGGG CGCGCGAAAG TCAAAAGTTA GACCAGGCTA GGAGAGTATA

       210        220        230        240        250
ATTAGTGGCA CTCCACCTCC AATGCCTCAC CAGCTGGTGT TTCAGATTAC
TAATCACCGT GAGGTGGAGG TTACGGAGTG GTCGACCACA AAGTCTAATG

       260        270        280        290        300
ATTAGCTATG TCTGGCAGAG GAAAGGGTGG AAAGGGTCTA GGCAAGGGTG
TAATCGATAC AGACCGTCTC CTTTCCCACC TTTCCCAGAT CCGTTCCCAC

       310        320        330        340        350
GCGCCAAGCG CCATCGCAAA GTCTTGCGTG ACAACATCCA GGGTATCACC
CGCGGTTCGC GGTAGCGTTT CAGAACGCAC TGTTGTAGGT CCCATAGTGG

       360        370        380        390        400
AAGCCCGCCA TCCGCCGCCT GGCTCGGCGC GGTGGGGTCA AGCGCATCTC
TTCGGGCGGT AGGCGGCGGA CCGAGCCGCG CCACCCCAGT TCGCGTAGAG

       410        420        430        440        450
CGGCCTCATC TACGAGGAGA CCCGTGGTGT GCTGAAGGTG TTCCTGGAGA
GCCGGAGTAG ATGCTCCTCT GGGCACCACA CGACTTCCAC AAGGACCTCT

       460        470        480        490        500
ACGTCATCCG CGACGCAGTC ACCTACACCG AGCACGCCAA GCGCAAGACC
TGCAGTAGGC GCTGCGTCAG TGGATGTGGC TCGTGCGGTT CGCGTTCTGG

       510        520        530        540        550
GTCACCGCTA TGGATGTGGT GTACGCTCTC AAGCGCCAGG GCCGCACCCT
CAGTGGCGAT ACCTACACCA CATGCGAGAG TTCGCGGTCC CGGCGTGGGA

       560        570        580        590        600
CTACGGCTTC GGAGGCTAGA CGCCGCCGCT TCAATTCCCC CCCCCCCCCC
GATGCCGAAG CCTCCGATCT GCGGCGGCGA AGTTAAGGGG GGGGGGGGGG
```

```
       610        620        630        640        650
ATCCCTAACG GCCCTTTTTA GGGCCAACCA CAGTCTCTTC AGGAGAGCTG
TAGGGATTGC CGGGAAAAAT CCCGGTTGGT GTCAGAGAAG TCCTCTCGAC

       660        670        680        690        700
ACACTGACTT GGGTCGTACA GGTAATAACC GCGGGTTTAG GACTCACGCT
TGTGACTGAA CCCAGCATGT CCATTATTGG CGCCCAAATC CTGAGTGCGA

       710        720        730        740        750
ACTAGGTGTT CCGCTTTTAG AGCCATCCAC TTAAGTTTCT ATACCACGGC
TGATCCACAA GGCGAAAATC TCGGTAGGTG AATTCAAAGA TATGGTGCCG

       760        770        780        790        800
GGATAGATAG CATCCAGCAG GGTCTGCTCA CACTGGGAAT TTTAATTCCT
CCTATCTATC GTAGGTCGTC CCAGACGAGT GTGACCCTTA AAATTAAGGA

       810        820        830        840        850
ACTTAGGGTG TGAGCTGGTT GTCAGGTCAA GAGACTGGCT AAGATTTTCT
TGAATCCCAC ACTCGACCAA CAGTCCAGTT CTCTGACCGA TTCTAAAAGA

       860        870        880        890        900
TTAGCTCGTT TGGAGCAGAA TTGCAATAAG GAGACCCTTT GGATGGGATG
AATCGAGCAA ACCTCGTCTT AACGTTATTC CTCTGGGAAA CCTACCCTAC

       910        920        930        940        950
ACCTATGTCC ACACATCAAA TGGCTATGTG GCTGTGTCCC TGTGTTTCCA
TGGATACAGG TGTGTAGTTT ACCGATACAC CGACACAGGG ACACAAAGGT

       960
ATGAGTGGCT GTGCTTGA
TACTCACCGA CACGAACT
```

The region of homology for both aforementioned sequences is shown below, where asterisks denote not homologous portions. Within the region shown, the first 118 base pairs have 80.5% homology and are used as a conserved DNA sequence probe in this example (sea urchin (top) base positions 449 to 567, mouse (bottom) base positions 257 to 375):

```
          *    *   * *  *     **  *  *   *  *  *  *   *           *
449     TAATGTCAGGCCGTGGTAAAGG-AGGCAA-GGGGCTCGG-AAAGGGAGGCGCCAAGCGTC
257     TA-TGTCTGGCAGAGG-AAAGGGTGG-AAAGGGTCTAGGCAA-GGGTGGCGCCAAGCGCC

               *   * *  *              *           *  *        *  **
        ATCGCAAGGTCCTACGAGACAACATCCAGGGCATCACCAAGCCTGCAATCCGCCGACT-C
        ATCGCAAAGTCTTGCGTGACAACATCCAGGGTATCACCAAGCCCGCCATCCGCCGCCTGG

                              **     *  * * *              *  **  *
        NNNNNNNNNNNNNNNNNNNNNNGAATCTCTGGTCTT-ATCTACGAGGAGACACGAGGG-G
        CTCGGCGCGGTGGGGTCAAGCG-CATCTCCGG-CCTCATCTACGAGGAGACCCG-TGGTG

        TGCTGAAGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
        TGCTGAAGGTGTTCCTGGAGAACGTCATCCGCGACGCAGTCACCTACACCGAGCACGCCA

                                                               * * *
        NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCCG-AAC-
        AGCGCAAGACCGTCACCGCTATGGATGTGGTGTACGCTCTCAAGCGCCAGGGCCGCACCC

        *  **          *     ***  *  * * **  ** *
        ACTGTACGGCTTCGGCGGCTAAGTGAAGCAGAC--TTGGCTA    780
        TCT--ACGGCTTCGGAGGCTA---GACGCCGCCGCTT--CAA    584
```

% = 84.503
F(342, 289) = .000E+00 E = .000

Restriction endonuclease cleavage sites were determined from the two sequences. A list of cleavage sites for the sea urchin and mouse sequences is shown below. Numbers indicate the 5' side of the cleavage site, unless site name is in brackets, which indicates that only the recognition site is known.

SEA URCHIN

Sequence	Appears at position		
AcyI (GPCGQC)	495		
AluI (AGCT)	147	267	
AsuI (GGNCC)	277	514	
AvaII (GGLCC)	514		
CauII (CCMGG)	276	377	
DdeI (CTNAG)	396	427	
DpnI (GATC)	326		
EcoRI* (PPATQQ)	184		
EcoRII (CCLGG)	531		
Fnu4HI (GCNGC)	254		
FnuDII (CGCG)	125	253	285
FokI (GGATG)	148		
FokI (CATCC)	324	515	
HaeII (PGCGCQ)	498		
HaeIII (GGCC)	256	279	459
HgaI (GCGTC)	491		
HgiCI (GGQPCC)	494		

HgiJII (GRGCYC)	483					
HhaI (GCGC)	125	253	295	497		
HindIII (AAGCTT)	145					
HinfI (GANTC)	200	431	561			
HpaII (CCGG)	275	376				
HphI (GGTGA)	368					
HphI (TCACC)	195	365	532			
MboI (GATC)	324					
MnlI (CCTC)	267	342				
MnlI (GAGG)	4	42	54	280	299	311
	372	463	484			
NarI (GGCGCC)	495					
NspBII (GCMGC)	256					
PvuI (CGATCG)	327					
ScrFI (CCNGG)	276	377	533			
SfaNI (GCATC)	409	527				
TaqI (TCGA)	117	327				

MOUSE

Sequence	Appears at position						
AcyI (GPCGQC)	302	571					
AflII (CTTAAG)	731						
AluI (AGCT)	234	256	648	815	855		
AsuI (GGNCC)	184	540	611	622			
AvaII (GGLCC)	184						
BssHII (GCGCGC)	162						
CauII (CCMGG)	135						
DdeI (CTNAG)	803	840					
DpnI (GATC)	190						
[EcoB] (AGCANNNNNNNNTCA)	766						
[EcoP1] (AGACC)	418	496	882				
[EcoP1] (GGTCT)	285	771					
[EcoP15] (CAGCAG)	765						
EcoRI (GAATTC)	2						
EcoRI* (PPATQQ)	4	790	845				
EcoRII (CCLGG)	338	368	443	536			
Fnu4HI (GCNGC)	366	543	574	577			
FnuDII (CGCG)	162	164	380	461	682		
FokI (GGATG)	526	905	910				
FokI (CATCC)	111	322	346	442	587	711	748
HaeII (PGCGCQ)	305	312	537				
HaeIII (GGCC)	404	542	612	624			
HgaI (GACGC)	472	579					

HsiAI	(GLGCLC)	485					
HsiCI	(GGQPCC)	21	301				
HsiJII	(GPGCQC)	135					
HhaI	(GCGC)	164	166	304	311	380	395
		494	536				
HinfI	(GANTC)	692					
HpaII	(CCGG)	78	135	401			
HphI	(TCACC)	220	339	462	495		
MboI	(GATC)	188					
MboII	(GAAGA)	105	124				
MboII	(TCTTC)	629					
MnlI	(CCTC)	202	227	236	415	559	
MnlI	(GAGG)	3	76	261	407	555	
NarI	(GGCGCC)	302					
NspBII	(GCMGC)	368	545	576	579		
PvuII	(CAGCTG)	234					
RsaI	(GTAC)	523	668				
SacII	(CCGCGG)	683					
ScrFI	(CCNGG)	135	340	370	445	538	
SfaNI	(GATGC)	127					
SfaNI	(GCATC)	385	751				
TaqI	(TCGA)	40					
Tth111I	(GACNNNGTC)	466					
Tth111II	(TGGTTG)	953					
XmnI	(GAANNNNTTC)	439					

The sea urchin and mouse sequences are compared with Hha I (GCGC) and the described probe sequence. The sea urchin sequence has cleavage sites at positions 295 and 497, thus creating a 202 bp fragment, which, if denatured, would hybridize with the probe sequence. Hha I (GCGC) sites in the mouse sequence, 166, 304, 311 and 380, indicate that fragments of 69 and 138 could be detected with the probe sequence.

Thus the genetic characterization for sea urchin is

202

while that for mouse is

69

138

Example 2

Use of trp D Gene of the Tryptophan Operon as a Probe

The same type of computer simulation as in Example 1 was carried out using trp D gene as a probe. This allows the conclusion that *E. coli* and *Salmonella typhimurium* can be distinguished by restriction fragments containing a conserved sequence.

The *E. coli* trp D gene with 684 bp's is shown below:

```
       10         20         30         40         50
GAAGCCGACG AAACCCGTAA CAAAGCTCGC GCCGTACTGC GCGCTATTGC
CTTCGGCTGC TTTGGGCATT GTTTCGAGCG CGGCATGACG CGCGATAACG

       60         70         80         90        100
CACCGCGCAT CATGCACAGG AGACTTTCTG ATGGCTGACA TTCTGCTGCT
GTGGCGCGTA GTACGTGTCC TCTGAAAGAC TACCGACTGT AAGACGACGA

      110        120        130        140        150
CGATAATATC GACTCTTTTA CGTACAACCT GGCAGATCAG TTGCGCAGCA
GCTATTATAG CTGAGAAAAT GCATGTTGGA CCGTCTAGTC AACGCGTCGT

      160        170        180        190        200
ATGGGCATAA CGTGGTGATT TACCGCAACC ATATACCGGC GCAAACCTTA
TACCCGTATT GCACCACTAA ATGGCGTTGG TATATGGCCG CGTTTGGAAT

      210        220        230        240        250
ATTGAACGCT TGGCGACCAT GAGTAATCCG GTGCTGATGC TTTCTCCTGG
TAACTTGCGA ACCGCTGGTA CTCATTAGGC CACGACTACG AAAGAGGACC

      260        270        280        290        300
CCCCGGTGTG CCGAGCGAAG CCGGTTGTAT GCCGGAACTC CTCACCCGCT
GGGGCCACAC GGCTCGCTTC GGCCAACATA CGGCCTTGAG GAGTGGGCGA

      310        320        330        340        350
TGCGTGGCAA GCTGCCCATT ATTGGCATTT GCCTCGGACA TCAGGCGATT
ACGCACCGTT CGACGGGTAA TAACCGTAAA CGGAGCCTGT AGTCCGCTAA

      360        370        380        390        400
GTCGAAGCTT ACGGGGGCTA TGTCGGTCAG GCGGGCGAAA TTCTCCACGG
CAGCTTCGAA TGCCCCCGAT ACAGCCAGTC CGCCCGCTTT AAGAGGTGCC

      410        420        430        440        450
TAAAGCCTCC AGCATTGAAC ATGACGGTCA GGCGATGTTT GCCGGATTAA
ATTTCGGAGG TCGTAACTTG TACTGCCAGT CCGCTACAAA CGGCCTAATT

      460        470        480        490        500
CAAACCCGCT GCCGGTGGCG CGTTATCACT CGCTGGTTGG CAGTAACATT
GTTTGGGCGA CGGCCACCGC GCAATAGTGA GCGACCAACC GTCATTGTAA

      510        520        530        540        550
CCGGCCGGTT TAACCATCAA CGCCCATTTT AATGGCATGG TGATGGCAGT
GGCCGGCCAA ATTGGTAGTT GCGGGTAAAA TTACCGTACC ACTACCGTCA

      560        570        580        590        600
ACGTCACGAT GCGGATCGCG TTTGTGGATT CCAGTTCCAT CCGGAATCCA
TGCAGTGCTA CGCCTAGCGC AAACACCTAA GGTCAAGGTA GGCCTTAGGT

      610        620        630        640        650
TTCTCACCAC CCAGGGCGCT CGCCTGCTGG AACAAACGCT GGCCTGGGCG
AAGAGTGGTG GGTCCCGCGA GCGGACGACC TTGTTTGCGA CCGGACCCGC

      660        670        680
CAGCATAAAC TAGAGCCAGC CAACACGCTG CAA
GTCGTATTTG ATCTCGGTCG GTTGTGCGAC GTT
```

The trp D gene, 683 base pairs, for <u>S. typhimurium</u> is shown below:

```
        10         20         30         40         50
GAAGCCGATG AAACCCGTAA TAAAGCGCGC GCCGTATTGC GTGCTATCGC
CTTCGGCTAC TTTGGGCATT ATTTCGCGCG CGGCATAACG CACGATAGCG

        60         70         80         90        100
CACCGCGCAT CATGCACAGG AGACCTTCTG ATGGCTGATA TTCTGCTGCT
GTGGCGCGTA GTACGTGTCC TCTGGAAGAC TACCGACTAT AAGACGACGA

       110        120        130        140        150
CGATAACATC GACTCGTTCA CTTGGAACCT GGCAGATCAG CTACGAACCA
GCTATTGTAG CTGAGCAAGT GAACCTTGGA CCGTCTAGTC GATGCTTGGT

       160        170        180        190        200
ACGGTCATAA CGTGGTGATT TACCGTAACC ATATTCCGGC GCAGACGCTT
TGCCAGTATT GCACCACTAA ATGGCATTGG TATAAGGCCG CGTCTGCGAA

       210        220        230        240        250
ATCGATCGCC TGGCGACAAT GAAAAATCCT GTGCTAATGC TCTCCCCCGG
TAGCTAGCGG ACCGCTGTTA CTTTTTAGGA CACGATTACG AGAGGGGGCC

       260        270        280        290        300
CCCGGGTGTT CCCAGCGAGG CAGGTTGTAT GCCGGAGCTG CTGACCCGAC
GGGCCCACAA GGGTCGCTCC GTCCAACATA CGGCCTCGAC GACTGGGCTG

       310        320        330        340        350
TACGCGGCAA GTTACCGATC ATCGGCATTT GTCTGGGGCA TCAGGCGATT
ATGCGCCGTT CAATGGCTAG TAGCCGTAAA CAGACCCCGT AGTCCGCTAA

       360        370        380        390        400
GTCGAAGCTT ACGGCGGTTA CGTCGGTCAG GCGGGAGAAA TCCTGCATGG
CAGCTTCGAA TGCCGCCAAT GCAGCCAGTC CGCCCTCTTT AGGACGTACC

       410        420        430        440        450
CAAAGCCTCC AGCATTGAGC ATGACGGTCA GGCGATGTTC GCCGGGCTCG
GTTTCGGAGG TCGTAACTCG TACTGCCAGT CCGCTACAAG CGGCCCGAGC
```

460 470 480 490 500
CGAATCCGCT ACCGGTCGCG CGTTATCATT CGCTGGTCGG CAGTAATGTT
GCTTAGGCGA TGGCCAGCGC GCAATAGTAA GCGACCAGCC GTCATTACAA

510 520 530 540 550
CCTGCCGGGC TGACCATTAA CGCCCATTTC AACGGCATGG TGATGGCGGT
GGACGGCCCG ACTGGTAATT GCGGGTAAAG TTGCCGTACC ACTACCGCCA

560 570 580 590 600
ACGTCATGAT GCGGATCGCG TTTGCGGTTT TCAATTTCAT CCCGAGTCCA
TGCAGTACTA CGCCTAGCGC AAACGCCAAA AGTTAAAGTA GGGCTCAGGT

610 620 630 640 650
TCCTGACGAC ACAGGGCGCG CGTCTACTGG AGCAAACATT AGCCTGGGCG
AGGACTGCTG TGTCCCGCGC GCAGATGACC TCGTTTGTAA TCGGACCCGC

660 670 680
CTGGCGAAGC TGGAACCGAC CAACACCCTA CAG
GACCGCTTCG ACCTTGGCTG GTTGTGGGAT GTC

Two homology regions between both sequences were next established, where the upper sequence if for <u>E. coli</u> and the lower one for <u>S.typhimurium</u>:

Region I

```
          **     *     *           *        *        **    * * *  **
452     AAACCCGCTGCCGGTGGCGCGTTATCACTCGCTGGTTGGCAGTAACATTCC-GGCCGG-T
453     AA-TCCGCTACCGGTCGCGCGTTATCATTCGCTGGTCGGCAGTAATGTTCCTGCC-GGGC

         * *    *           *  *              *        *
        TTAACCATCAACGCCCATTTTAATGGCATGGTGATGGCAGTACGTCACGATGCGGATCGC
        TGA-CCATTAACGCCCATTTCAACGGCATGGTGATGGCGGTACGTCATGATGCGGATCGC

             *  **   * *  *     *  *     *  *  *  *        *  *  *
       /GTTTGTGG-ATTCCAGTTCCATCCGGAATCCATTCTCACCACCCAGGGGGGTGGCTGCT
        GTTTGCGGTTTTC-AATTTCATCCCGAGTCCATCCTGACGACACAGGGCGCGCGTCTACT

           *     **  *         **  **  *  *  *  * * *     *  *
        GGAACAAACGCTGGCCTGGGCGC-AGCATAAACTAGAGCC-AGCCAACACGCTGCA    682
        GGAGCAAACATTAGCCTGGGCGCTGGC-GAAGCTGGAACCGACC-AACACCCTACA    682
```

% = 78.390
P(236, 185) = .000E+00   E = .000

Region II

```
               *           **  **  *   **   *    *     *
1      GAAGCCGACGAAACCCGTAA-CAA-AGCTCGCGCCGTACTGCGCTATTGCCACCGCGC
1      GAAGCCGATGAAACCCGTAATAAAGCGCGC--CGTATTGCGTGCTATCGCCACCGCGC

                       *  *           *                *        *
       ATCATGCACAGGAGAC-TTTCTGATGGCTGACATTCTGCTGCTCGATAATATCGACTC-T
       ATCATGCACAGGAGACCTT-CTGATGGCTGATATTCTGCTGCTCGATAACATCGACTCGT

        **  ** * * * *           * * * * *  **  *
       TTTAC--GTACAACCTGGCAGATCAGTTGCGCAGC-AATGGG-CATAACGTGGTGATTTA
       T-CACTTGGA-ACC-TGGCAGATCAGCTACG-AACCAA-CGGTCATAACGTGGTGATTTA

          *        *        *  *    * *  *   * * *    *    **  *
       CCGCAACCATATACCGGCGCAAAC-CTTAATTGAACGC-TTGGCGACCATGAGTAA-TCC
       CCGTAACCATATTCCGGCGCAGACGCTTA-TCGATCGCCTGG-CGACAATGA-AAAATCC

       *     *     * * *  *     *   *   **  * * ** *  *
       GGTGCTGATGCT-TTCTCCTGGCCCCGG-TGT-GCC-GAGCGAAGCCGGTTGTATGCCGG
       TGTGCTAATGCTCTCC-CCCGGCCC-GGGTGTTCCCAGCG--AGGCAGGTTGTATGCCGG

        *  *  *     *  **   **     * *  *  *  *        *  **  *
       AACTCCTCACCCG-CT-TGCGTGGCAAGCTGCCCATTATTGGCATTTGCCT-CGGACATC
       AGCTGCTGACCCGACTACGCG--GCAAGTTACCGATCATCGGCATTTGTCTGGGG-CATC

                             *  **  *              *     *  *  *  *
       AGGCGATTGTCGAAGCTTACGG-GGGCTATGTCGGTCAGGCGGGCGAAATTCTCCACGG-
       AGGCGATTGTCGAAGCTTACGGCGG-TTACGTCGGTCAGGCGGGAGAAATCCTGCATGGC

       *  *              *                    *
       TAAAGCCTCCAGCATTGAACATGACGGTCAGGCGATGTTTGCCGG    445
       AAA-GCCTCCAGCATTGAGCATGACGGTCAGGCGATGTTCGCCGG    445
```

% = 80.215
P(465, 373) = .000E+00   E = .000

Restriction sites in both sequences are shown as follows:

E. coli

paII	(CCGG)	187	229	254	272	283	443	463	502
		506	592						
phI	(GGTGA)	177	552						
phI	(TCACC)	285	597						
boI	(GATC)	135	564						

S. typhimurium

aII	(CCGG)	187	248	253	283	443	463	506
hI	(GGTGA)	177	552					
oI	(GATC)	135	204	317	564			
lI	(CCTC)	417						

The E. coli sequence has Mbo I (GATC) sites at 135 and 564. There is a 429 bp fragment that can be detected by both Region 1 and 2 probes. The same enzyme has sites at 135, 204, 317, and 564 in the S. typhimurium sequence. A probe of the two homology regions would detect fragments of 69, 113 and 247 bp.

Thus the identifying genetic characterization for E. coli with this probe and enzyme is

429

whereas that for S. typhimurium is

69

113

247

Example 3

Use of α-Fetoprotein Gene as a Probe

This example shows the use of a region of homology in the α-fetoprotein gene sequence of human and rat, and the endonuclease MnlI (GAGG).

The human α-fetoprotein message cDNA (1578 bp's) is as follows:

```
         10         20         30         40         50
AGCTTGGCAT AGCTACCATC ACCTTTACCC AGTTTGTTCC GGAAGCCACC
TCGAACCGTA TCGATGGTAG TGGAAATGGG TCAAACAAGG CCTTCGGTGG

         60         70         80         90        100
GAGGAGGAAG TGAACAAAAT GACTAGCGAT GTGTTGGCTG CAATGAAGAA
CTCCTCCTTC ACTTGTTTTA CTGATCGCTA CACAACCGAC GTTACTTCTT

        110        120        130        140        150
AAACTCTGGC GATGGGTGTT TAGAAAGCCA GCTATCTGTG TTTCTGGATG
TTTGAGACCG CTACCCACAA ATCTTTCGGT CGATAGACAC AAAGACCTAC

        160        170        180        190        200
AAATTTGCCA TGAGACGGAA CTCTCTAACA AGTATGGACT CTCAGGCTGC
TTTAAACGGT ACTCTGCCTT GAGAGATTGT TCATACCTGA GAGTCCGACG

        210        220        230        240        250
TGCAGCCAAA GTGGAGTGGA AAGACATCAG TGTCTGCTGG CACGCAAGAA
ACGTCGGTTT CACCTCACCT TTCTGTAGTC ACAGACGACC GTGCGTTCTT

        260        270        280        290        300
GACTGCTCCG GCCTCTGTCC CACCCTTCCA GTTTCCAGAA CCTGCCGAGA
CTGACGAGGC CGGAGACAGG GTGGGAAGGT CAAAGGTCTT GGACGGCTCT

        310        320        330        340        350
GTTGCAAAGC ACATGAAGAA AACAGGGCAG TGTTCATGAA CAGGTTCATC
CAACGTTTCG TGTACTTCTT TTGTCCCGTC ACAAGTACTT GTCCAAGTAG

        360        370        380        390        400
TATGAAGTGT CAAGGAGGAA CCCCTTCATG TATGCCCCAG CCATTCTGTC
ATACTTCACA GTTCCTCCTT GGGGAAGTAC ATACGGGGTC GGTAAGACAG

        410        420        430        440        450
CTTGGCTGCT CAGTACGACA AGGTCGTTCT GGCATGCTGC AAAGCTGACA
GAACCGACGA GTCATGCTGT TCCAGCAAGA CCGTACGACG TTTCGACTGT
```

```
       460        470        480        490        500
ACAAGGAGGA GTGCTTCCAG ACAAAGAGAG CATCCATTGC AAAGGAATTA
TGTTCCTCCT CACGAAGGTC TGTTTCTCTC GTAGGTAACG TTTCCTTAAT

       510        520        530        540        550
AGAGAAGGAA GCATGTTAAA TGAGCATGTA TGTTCAGTGA TAAGAAAATT
TCTCTTCCTT CGTACAATTT ACTCGTACAT ACAAGTCACT ATTCTTTTAA

       560        570        580        590        600
TGGATCCCGA AACCTCCAGG CAACAACCAT TATTAAGCTA AGTCAAAAGT
ACCTAGGGCT TTGGAGGTCC GTTGTTGGTA ATAATTCGAT TCAGTTTTCA

       610        620        630        640        650
TAACTGAAGC AAATTTTACT GAGATTCAGA AGCTGGCCCT GGATGTGGCT
ATTGACTTCG TTTAAAATGA CTCTAAGTCT TCGACCGGGA CCTACACCGA

       660        670        680        690        700
CACATCCACG AGGAGTGTTG CCAAGGAAAC TCGCTGGAGT GTCTGCAGGA
GTGTAGGTGC TCCTCACAAC GGTTCCTTTG AGCGACCTCA CAGACGTCCT

       710        720        730        740        750
TGGGGAAAAA GTCATGACAT ATATATGTTC TCAACAAAAT ATTCTGTCAA
ACCCCTTTTT CAGTACTGTA TATATACAAG AGTTGTTTTA TAAGACAGTT

       760        770        780        790        800
GCAAAATAGC AGAGTGCTGC AAATTACCCA TGATCCAACT AGGCTTCTGC
CGTTTTATCG TCTCACGACG TTTAATGGGT ACTAGGTTGA TCCGAAGACG

       810        820        830        840        850
ATAATTCACG CAGAGAATGG CGTCAAACCT GAAGGCTTAT CTCTAAATCC
TATTAAGTGC GTCTCTTACC GCAGTTTGGA CTTCCGAATA GAGATTTAGG

       860        870        880        890        900
AAGCCAGTTT TTGGGAGACA GAAATTTTGC CCAATTTTCT TCAGAGGAAA
TTCGGTCAAA AACCCTCTGT CTTTAAAACG GGTTAAAAGA AGTCTCCTTT

       910        920        930        940        950
AAATCATGTT CATGGCAAGC TTTCTTCATG AATACTCAAG AACTCACCCC
TTTAGTACAA GTACCGTTCG AAAGAAGTAC TTATGAGTTC TTGAGTGGGG

       960        970        980        990       1000
AACCTTCCTG TCTCAGTCAT TCTAAGAATT GCTAAAACGT ACCAGGAAAT
TTGGAAGGAC AGAGTCAGTA AGATTCTTAA CGATTTTGCA TGGTCCTTTA

      1010       1020       1030       1040       1050
ATTGGAGAAG TGTTCCCAGT CTGGAAATCT ACCTGGATGT CAGGACAATC
TAACCTCTTC ACAAGGGTCA GACCTTTAGA TGGACCTACA GTCCTGTTAG
```

```
      1060       1070       1080       1090       1100
TGGAAGAAGA ATTGCAGAAA CACATCGAGG AGAGCCAGGC ACTGTCCAAG
ACCTTCTTCT TAACGTCTTT GTGTAGCTCC TCTCGGTCCG TGACAGGTTC

      1110       1120       1130       1140       1150
CAAAGCTGCG CTCTCTACCA GACCTTAGGA GACTACAAAT TACAAAATCT
GTTTCGACGC GAGAGATGGT CTGGAATCCT CTGATGTTTA ATGTTTTAGA

      1160       1170       1180       1190       1200
GTTCCTTATT GGTTACACGA GGAAAGCCCC TCAGCTGACC TCAGCAGAGC
CAAGGAATAA CCAATGTGCT CCTTTCGGGG AGTCGACTGG AGTCGTCTCG

      1210       1220       1230       1240       1250
TGATCGACCT CACCGGGAAG ATGGTGAGCA TTGCCTCCAC GTGCTGCCAG
ACTAGCTGGA GTGGCCCTTC TACCACTCGT AACGGAGGTG CACGACGGTC

      1260       1270       1280       1290       1300
CTCAGCGAGG AGAAATGGTC CGGCTGTGGT GAGGGAATGG CCGACATTTT
GAGTCGCTCC TCTTTACCAG GCCGACACCA CTCCCTTACC GGCTGTAAAA

      1310       1320       1330       1340       1350
CATTGGACAT TTGTGTATAA GGAATGAAGC AAGCCCTGTG AACTCTGGTA
GTAACCTGTA AACACATATT CCTTACTTCG TTCGGGACAC TTGAGACCAT

      1360       1370       1380       1390       1400
TCAGCCACTG CTGCAACTCT TCGTATTCCA ACAGGAGGCT ATGCATCACC
AGTCGGTGAC GACGTTGAGA AGCATAAGGT TGTCCTCCGA TACGTAGTGG

      1410       1420       1430       1440       1450
AGTTTTCTGA GGGATGAAAC CTATGCCCCT CCCCCATTCT CTGAGGATAA
TCAAAAGACT CCCTACTTTG GATACGGGGA GGGGGTAAGA GACTCCTATT

      1460       1470       1480       1490       1500
ATTCATCTTC CACAAGGATC GTGCCAAGCT CGGCAAAGCC CTACAGACCA
TAAGTAGAAG GTGTTCCTAG CACGGTTCGA GCCGTTTCGG GATGTCTGGT

      1510       1520       1530       1540       1550
TGAAACAAGA GCTTCTCATT AACCTGGTGA AGCAAAAGCC TGAACTGACA
ACTTTGTTCT CGAAGAGTAA TTGGACCACT TCGTTTTCGG ACTTGACTGT

      1560       1570
GAGGAGCAGC TGGCGGCTGT CACTGCAG
CTCCTCGTCG ACCGCCGACA GTGACGTC
```

The rat α-fetoprotein 3' end cDNA is as follows (540 bp's):

```
        10         20         30         40         50
GAGGGACTGG CCGACATTTA CATTGGACAC TTGTGTTTAA GACATGAGGC
CTCCCTGACC GGCTGTAAAT GTAACCTGTG AACACAAATT CTGTACTCCG

        60         70         80         90        100
AAACCCTGTG AACTCCGGTA TCAACCACTG CTGCAGTTCC TCGTATTCCA
TTTGGGACAC TTGAGGCCAT AGTTGGTGAC GACGTCAAGG AGCATAAGGT

       110        120        130        140        150
ACAGGAGGCT CTGCATCACC AGCTTTCTGA GGGACGAAAC CTACGTCCCT
TGTCCTCCGA GACGTAGTGG TCGAAAGACT CCCTGCTTTG GATGCAGGGA

       160        170        180        190        200
CTACCATTCT CTGCGACAAA TTCATCTTCC ACAAGGAATC TGTGCCAAGC
GATGGTAAGA GACGCTGTTT AAGTAGAAGG TGTTCCTTAG ACACGGTTCG

       210        220        230        240        250
TCAGGGCCGA GCACCACAGA CCATGAAACA AGAGCTTCTC ATTAACCTAG
AGTCCCGGCT CGTGGTGTCT GGTACTTTGT TCTCGAAGAG TAATTGGATC

       260        270        280        290        300
TGAAACAAAA GCCTGAAATG ACAGAGGAGC AGCACGCGGC TGTCACTGCT
ACTTTGTTTT CGGACTTTAC TGTCTCCTCG TCGTGCGCCG ACAGTGACGA

       310        320        330        340        350
GATTTCTCTG GCCTCTTGGA GAAGTGCTGC AAAGACCAGG ATCAGGAAGC
CTAAAGAGAC CGGAGAACCT CTTCACGACG TTTCTGGTCC TAGTCCTTCG

       360        370        380        390        400
CTGTTTCGCA AAAGAGGTCC AAGTTGATTT CCAAACTCGT GAGGCTTTGG
GACAAAGCGT TTTCTCCAGG TTCAACTAAA GGTTTGAGCA CTCCGAAACC

       410        420        430        440        450
GGGTTTAAAC ATCTCCAAGA GGAAGAAAGG ACAAAAAAAT GTGTCGACGC
CCCAAATTTG TAGAGGTTCT CCTTCTTTCC TGTTTTTTTA CACAGCTGCG

       460        470        480        490        500
TTTGGTGTGA GCTTTTCGGT TTGATGGTAA CTGGTGGAGA CTTCCATGTG
AAACCACACT CGAAAAGCCA AACTACCATT GACCACCTCT GAAGGTACAC

       510        520        530        540
GGATTTCTAT GCCTAAGGAA TAAAGACTTT TCAACTGTTA
CCTAAAGATA CGGATTCCTT ATTTCTGAAA AGTTGACAAT
```

The homologous regions between both is as follows, (human: upper; rat: lower):

```
            ***                  *           *   *        *        *
1367    CTCTTCGTATTCCAACAGGAGGCTATGCATCACCAG-TTTTCTGAGGGATGAAACCTATG
  90    CTC---GTATTCCAACAGGAGGCTCTGCATCACCAGCTTT-CTGAGGGACGAAACCTACG

        *     **  *        **  **  *                 *  *
        CCCCTC-CCCCATTCTCTGAGGA-TAAATTCATCTTCCACAAGGA-TC-GTGCCAAGCTC
        TCCCTCTACC-ATTCTCTG-CGACAAA-TTCATCTTCCACAAGGAATCTGTGCCAAGCTC

        *  ** * *  *  *                                 *     *
        -GG--CAAAGC-CCTACAGACCATGAAACAAGAGCTTCTCATTAACCTGGTGAAGCAAAA
        AGGGCCGA-GCACC-ACAGACCATGAAACAAGAGCTTCTCATTAACCTAGTGAAACAAAA

               *               **
        GCCTGAACTGACAGAGGAGCAGCTGGCGGCTGTCACTGC     1576
        GCCTGAAATGACAGAGGAGCAGCACGCGGCTGTCACTGC      299

% = 85.845
P( 219, 188) = .000E+00       E = .000
```

Restriction sites for both human and rat are as follows:

HUMAN

MboII	(GAAGA)	108	261	328	1066	1069	1230		
MboII	(TCTTC)	881	916	1361	1449				
MnlI	(CCTC)	273	574	1190	1200	1219	1245	1439	
MnlI	(GAGG)	44	47	358	449	653	887	1070	1162
		1250	1274	1378	1402	1436	1544		

RAT

MboII	(GAAGA)	435						
MboII	(TCTTC)	168						
MnlI	(CCTC)	100	159	323				
MnlI	(GAGG)	39	98	122	267	357	384	412

It can be calculated that fragments containing a portion of the conserved sequence, 24, 34, 104 and 108 bp describe the human DNA. Fragments of 24, 59, 90 and 145 describe the rat DNA. While both sequences contain the 24 bp fragment, it is the set of fragments (taxonomic characters) that is of significance.

CLAIMS

1. A method of characterizing an unknown organism species which comprises:

determining the position of part or whole of evolutionarily conserved sequences in genetic material of said organism relative to a known position in said genetic material (other than by determining the chromatographic pattern of restriction endonuclease digested DNA from said known organism, which digested DNA has been hybridized or reassociated with ribosomal RNA information-containing nucleic acid from or derived from a known probe organism), thereby to obtain an identifying genetic characterization of said unknown organism, and

comparing said characterization with information from at least two sets of identifying genetic characterizations derived from the same conserved sequences, each of said sets defining a known organism species.

2. A method as claimed in Claim 1, wherein the known position is defined by one or more restriction endonuclease cleavage sites.

3. A method as claimed in Claim 1, wherein the genetic material is DNA.

4. A method as claimed in Claim 1, which comprises comparing the chromatographic pattern of restriction endonuclease digested DNA from said unknown organism, which digested DNA has been hybridized or reassociated with

conserved DNA sequence information-containing nucleic acid from or derived from a probe organism or from a consensus sequence, with equivalent chromatographic patterns of at least two known different organism species.

5. A method as claimed in Claim 4, wherein said conserved DNA information-containing nucleic acid is detectably labelled.

6. A method as claimed in Claim 5, wherein said conserved DNA information-containing nucleic acid is radiolabelled or metal labelled.

7. A method as claimed in Claim 4, 5 or 6, wherein said conserved DNA information-containing nucleic acid probe is an RNA probe.

8. A method as claimed in Claim 4, 5 or 6, wherein said conserved DNA information-containing nucleic acid probe is DNA complementary to RNA.

9. A method as claimed in Claim 4, 5 or 6, wherein said conserved DNA information-containing nucleic acid probe is DNA obtained by nick-translating or cloning DNA complementary to RNA.

10. A method as claimed in any one of Claims 1 to 4, wherein said unknown organism being characterized is a cell or cells of a strain in an in vitro culture.

11. A method as claimed in any one of claims 1 to 4, wherein said unknown organism being characterized and said probe organism are both from the same kingdom, subkingdom,

division, subdivision, phylum, subphylum, class, subclass, order, family, tribe or genus.

12. A method as claimed in Claim 4, wherein said unknown organism being characterized and said probe organism are both eukaryotic.

13. A method as claimed in Claim 4, wherein said unknown organism being characterized and said probe organism are both prokaryotic.

14. A method as claimed in Claim 4, wherein said unknown organism being characterized is eukaryotic and said probe organism is prokaryotic.

15. A method as claimed in Claim 12 or 14, which further comprises detecting for the presence of a nucleic acid sequence or sequences creating a taxon below the rank of species or an infrasubspecific subdivision.

16. A method as claimed in Claim 4, wherein said unknown organism being characterized is prokaryotic and said probe organism is eukaryotic.

17. A method as claimed in Claim 13 or 16, wherein said prokaryotic organism being characterized is selectively being detected while in the presence of a eukaryotic organism.

18. A method as claimed in Claim 17, wherein said prokaryotic organism is a bacterium.

19. A method as claimed in Claim 12, wherein the DNA from said eukaryotic organism being characterized is

nuclear DNA, and the conserved DNA information-containing nucleic acid from said eukaryotic probe organism is not derived from mitochondria or chloroplasts.

20. A method as claimed in Claim 12, wherein the DNA from said eukaryotic organism being characterized is mitochondrial DNA and the conserved DNA information-containing nucleic acid from said eukaryotic probe organism is derived from mitochondria or chloroplasts.

21. A method as claimed in Claim 12, wherein DNA from said eukaryotic organism being characterized is chloroplast DNA and the conserved DNA information-containing nucleic acid from said eukaryotic probe organism is derived from mitochondria or chloroplasts.

22. A method as claimed in Claim 14, wherein said DNA from said eukaryotic organism being characterized is derived from mitochondrial DNA.

23. A method as claimed in Claim 14, wherein said DNA from said eukaryotic organism being characterized is derived from chloroplast DNA.

24. A method as claimed in Claim 16, wherein said conserved DNA information-containing nucleic acid probe is derived from mitochondria or from chloroplasts.

25. A method as claimed in Claim 12 or 14, which further comprises identifying in said unknown eukaryotic organism being characterized a virus, or a virus-derived DNA creating a taxon below the rank of species.

26. A method of identifying a bacterium of an unknown bacterial strain present in a sample which comprises:

determining the position of part or whole of evolutionarily conserved sequences in DNA of said bacterium, relative to a known position in said DNA (other than by determining the chromatographic pattern of restriction endonuclease digested DNA from said unknown organism, which digested DNA has been hybridized or reassociated with ribosomal RNA information-containing nucleic acid from or derived from a known probe organism), thereby to obtain an identifying genetic characterization of said unknown bacterium, and

comparing said characterization with information from at least two sets of identifying genetic characterizations derived from the same conserved sequences, each of said sets defining a known bacterial species.

27. A method as claimed in Claim 26, wherein the known position is defined by one or more restriction endonuclease cleavage sites.

28. A method as claimed in Claim 26, which comprises comparing the chromatographic pattern of restriction-endonuclease digested DNA from said unknown bacterium, which digested DNA has been hybridized or reassociated with conserved DNA sequence information-containing nucleic acid from or derived from a probe bacterium or from a consensus sequence, with equivalent chromatographic patterns of known bacteria.

29. A method as claimed in Claim 26, wherein said unknown bacterium is present in a fermentation medium or in a secretion or excretion product.

30. A method as claimed in Claim 26, wherein said unknown bacterium is present in or associated with eukaryotic tissue.

31. A method as claimed in Claim 30, wherein said bacterium is present in or associated with animal or plant cells.

32. A method as claimed in Claim 30, wherein said bacterium is present in or associated with human cells, or associated with plant root cells.

33. A method as claimed in any one of Claims 28 to 32, wherein said conserved DNA information-containing nucleic acid from said probe bacterium is detectably labelled.

34. A method as claimed in Claim 33, wherein said label is a radiolabel or a metal label.

35. A method as claimed in Claim 33, wherein said nucleic acid from said probe bacterium is RNA.

36. A method as claimed in Claim 33, wherein said nucleic acid from said probe bacterium is complementary DNA to RNA.

37. A method as claimed in any one of Claims 28 to 32, wherein said unknown bacterium is pathogenic towards plants or animals.

38. A method as claimed in any one of Claims 28 to 32, which further comprises detecting for the presence of a nucleic acid sequence or sequences creating a taxon below the rank of species, or an infrasubspecific subdivision.

39. A method as claimed in Claim 38, wherein said nucleic acid sequence or sequences are all or part of a bacteriophage genome.

40. A method as claimed in Claim 38, wherein said nucleic acid sequence or sequences are all or part of an extrachromosomal genetic element, a plasmid, or an episome.

41. A method as claimed in Claim 38, wherein said sequence or sequences code for an R-factor or for an antibiotic resistance factor.

42. A method as claimed in Claim 28, wherein said chromatographic patterns of known bacteria are present in a catalog containing patterns for at least two different bacteria.

43. A kit comprising a carrier being compartmentalized to receive in close confinement therein one or more container means, wherein a first container means contains conserved genetic material sequence information-containing nucleic acid (other than ribosomal RNA information-containing nucleic acid) from or derived from a probe organism or from a consensus sequence; and wherein said kit also contains a catalog having hybridized or ..

reassociated chromatographic band patterns for at least two known different organism species, or said kit contains at least two known organism species, or genetic material derived therefrom.

44. A kit as claimed in Claim 43, wherein the genetic material is DNA.

45. A kit as claimed in Claim 43 or 44, which also comprises a second container means containing one or more restriction endonuclease enzymes.

46. A kit as claimed in Claim 44, wherein the conserved DNA sequence information-containing nucleic acid probe is detectably labelled.

47. A kit comprising a carrier being compartmentalized to receive in close confinement therein one or more container means, wherein a first container means contains conserved DNA sequence information-containing nucleic acid (other than ribosomal RNA information-containing nucleic acid) from or derived from a probe organism or from a consensus sequence, said nucleic acid being in detectably labelled form; and

wherein said kit also comprises a second container means containing one or more restriction endonuclease enzymes.

48. A kit as claimed in Claim 43, 45 or 47, wherein said conserved DNA sequence information-containing nucleic acid probe is RNA.

49. A kit as claimed in Claim 43, 45 or 47, wherein said conserved DNA sequence information-containing nucleic acid probe is DNA complementary to RNA.

50. A kit as claimed in Claim 48, which also comprises a container means containing one or more detectably labelled deoxynucleoside triphosphates.

51. A kit as claimed in Claim 43, 45 or 47, wherein said probe organism is a prokaryote.

52. A kit as claimed in Claim 43, 45 or 47, wherein said probe organism is a eukaryote.

53. A kit as claimed in Claim 51, wherein said prokaryote is a bacterium.

54. A kit as claimed in Claim 52, wherein said nucleic acid probe of said eukaryote is derived from an organelle thereof.

55. A kit as claimed in Claim 49, wherein said cDNA is labelled with ^{32}P, ^{14}C or ^{3}H.

56. A kit as claimed in Claim 49, wherein said cDNA is a faithful copy of the RNA it is derived from.

57. A kit as claimed in Claim 43, 45 or 47, which also comprises one or more container means containing viral nucleic acid probes.

58. A kit as claimed in Claim 43, 45 or 47 wherein said catalog is a book, a computer tape, a computer disk, or a computer memory.

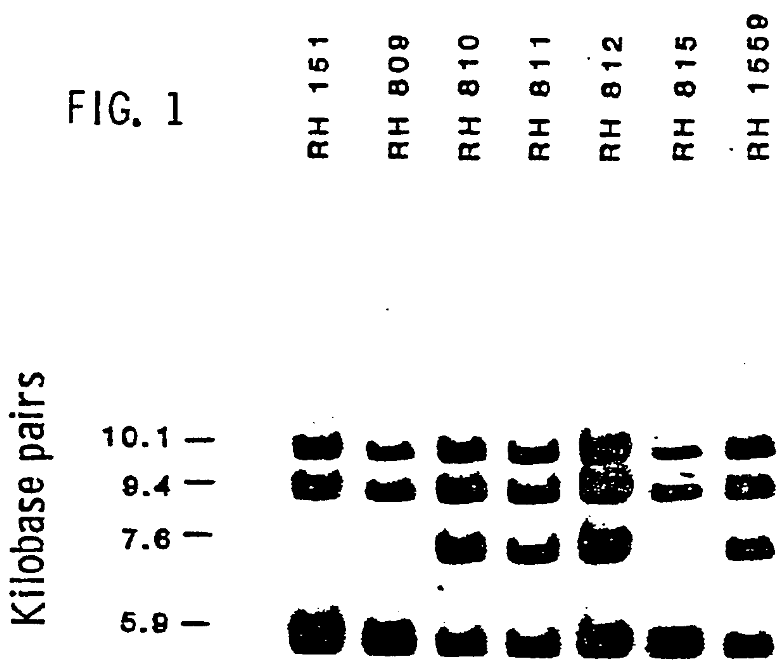
59. A kit as claimed in Claim 43, 45 or 47, wherein

said catalogue also includes viral chromatographic patterns.

60. A kit as claimed in Claim 43, 45 or 47 wherein said catalogue also includes patterns for infrasubspecific taxons.

EcoR I digested DNA
from P. aeruginosa strains

FIG. 1



PST I digested DNA
from *P. aeruginosa* strains

FIG. 2

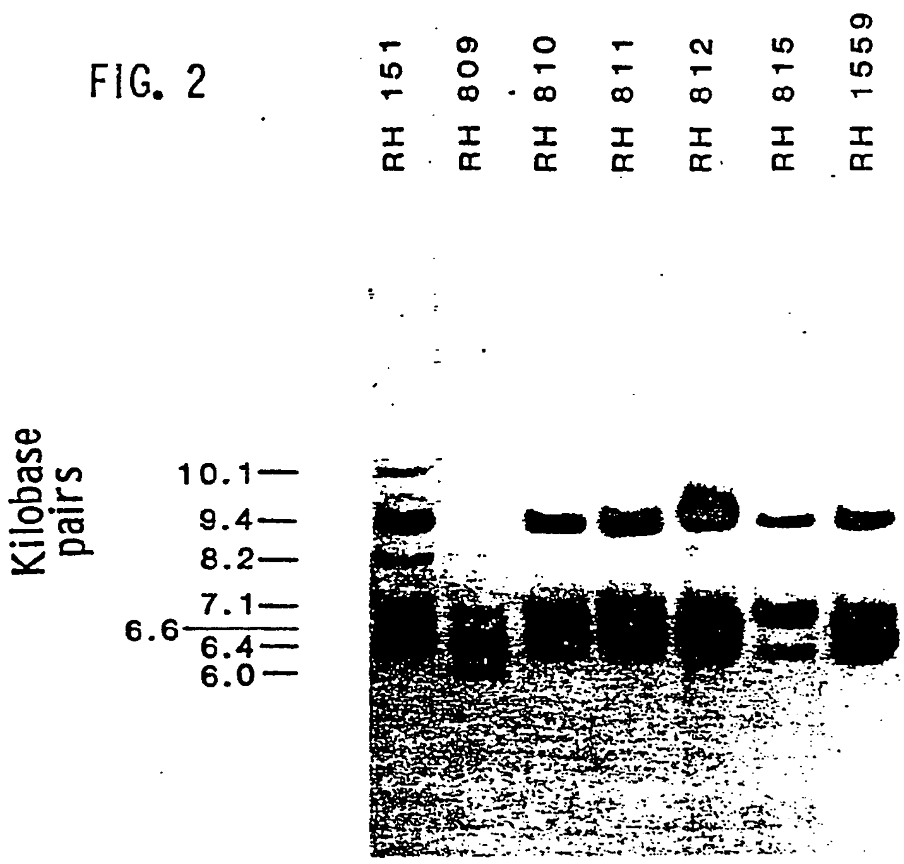


FIG. 3 EcoR I digested DNA from *Pseudomonas* and *Acinetobacter* species

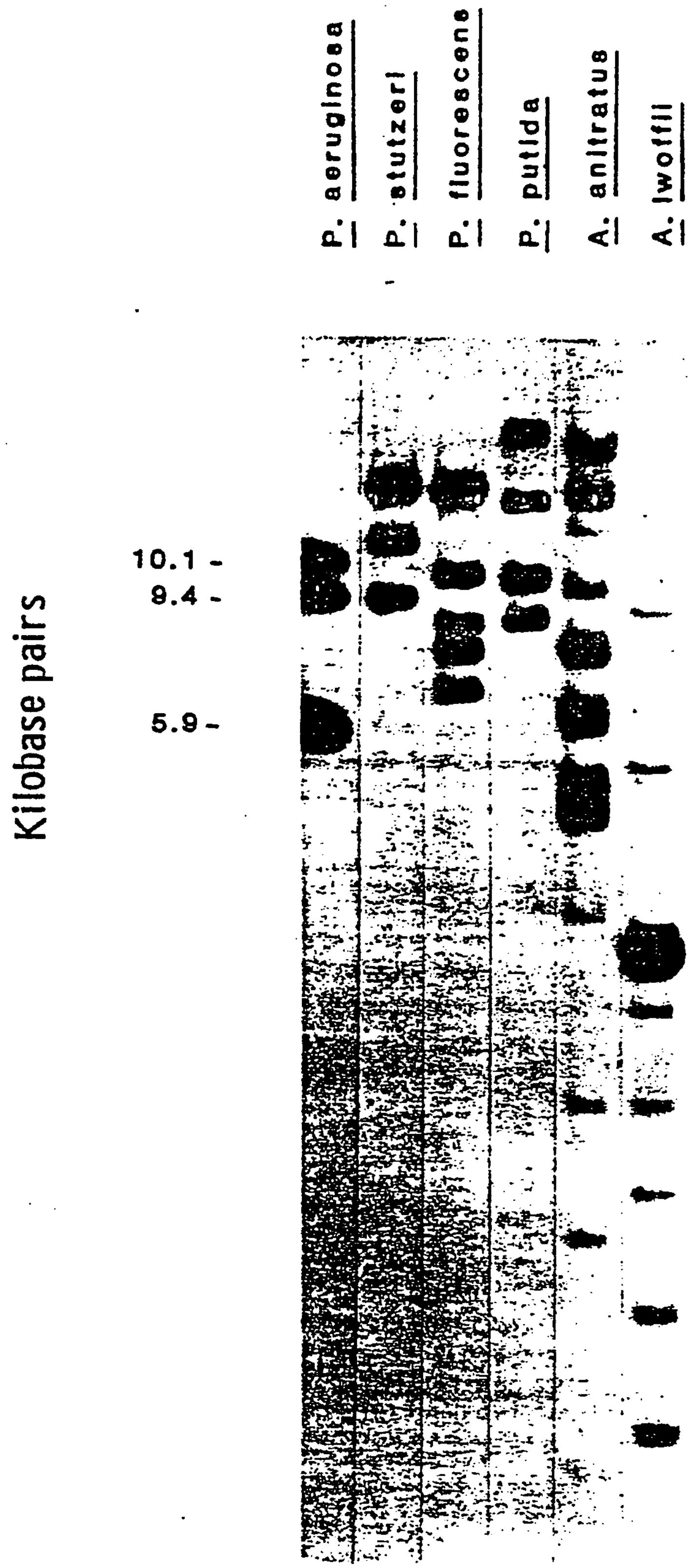
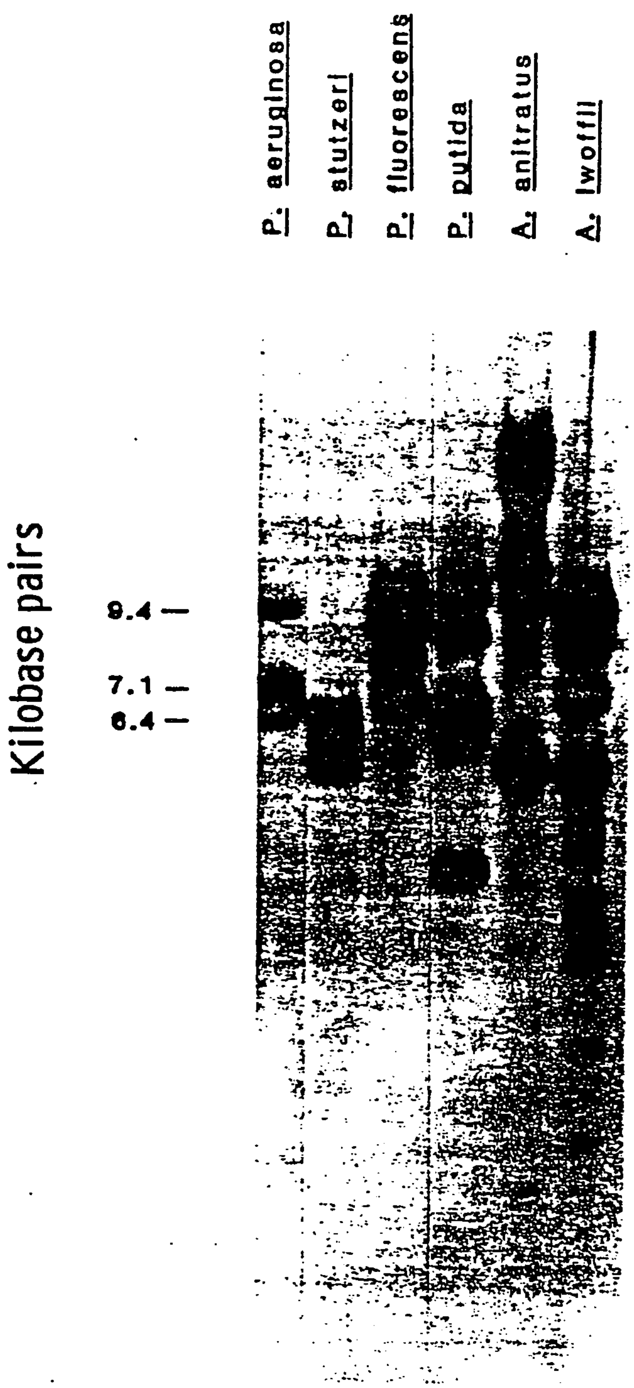
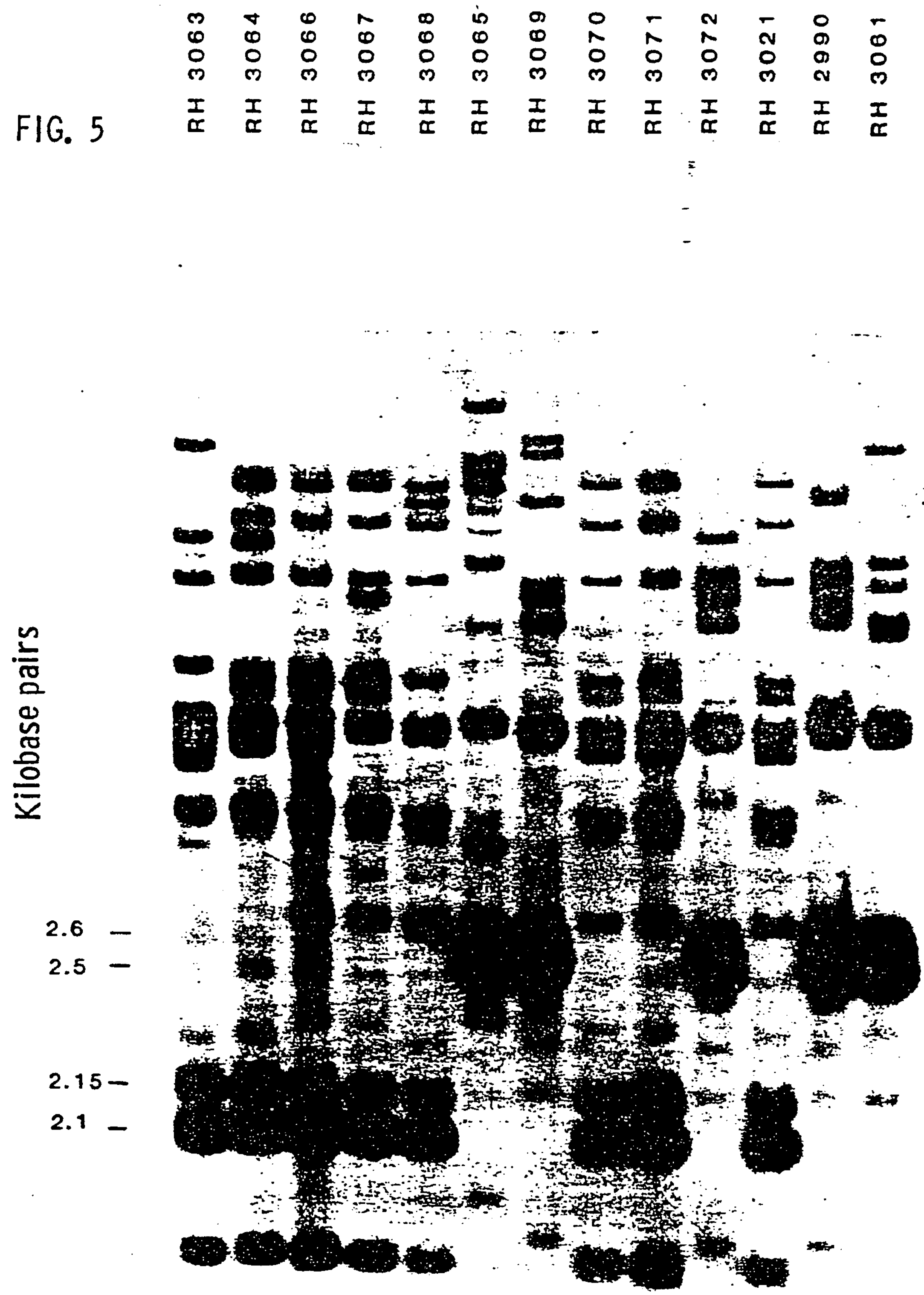


FIG. 4 PST I digested DNA from *Pseudomonas* and *Acinetobacter* species



EcoR I digested DNA from Bacillus subtilis strains

FIG. 5



PST I digested DNA from Bacillus subtilis strains

FIG. 6

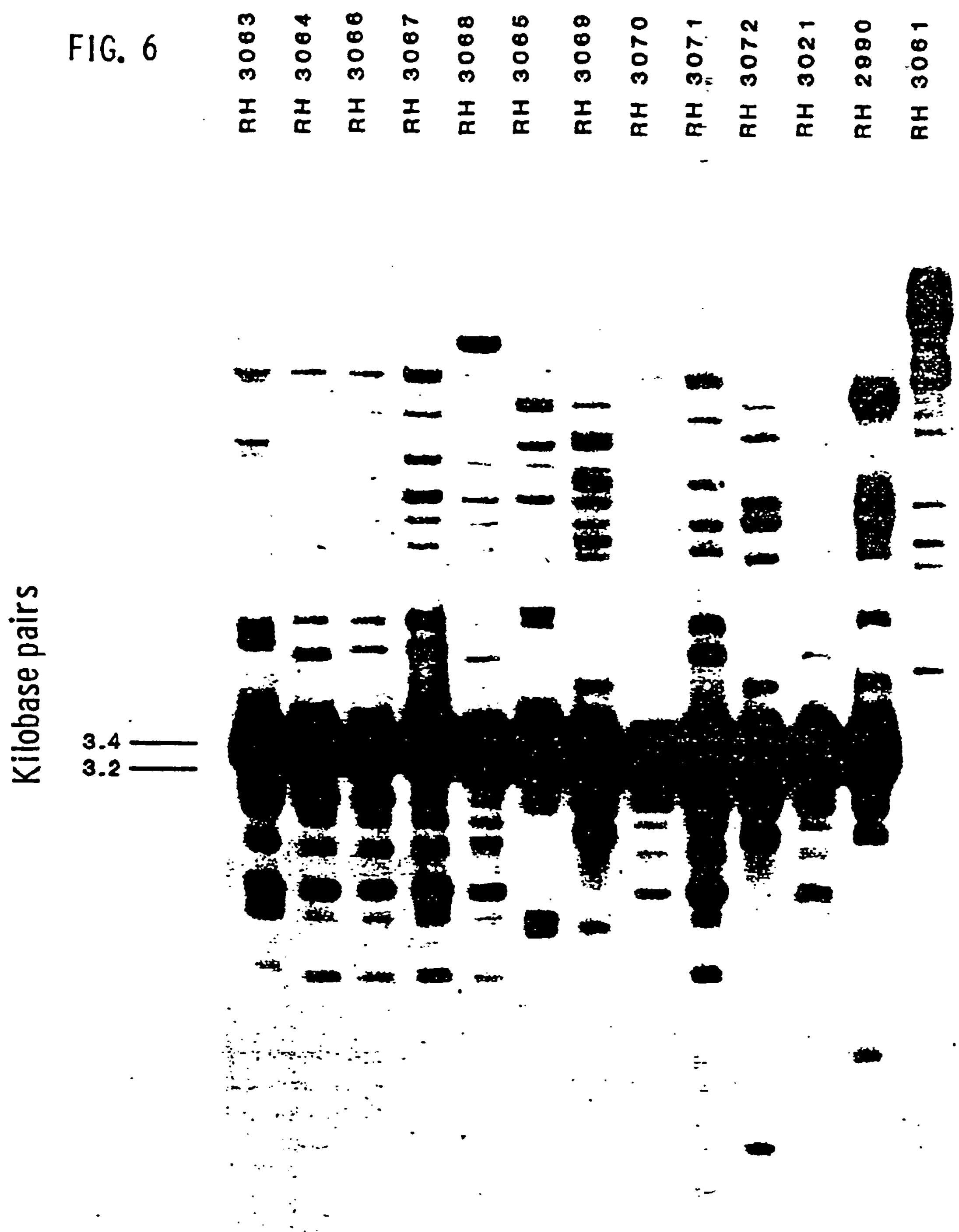
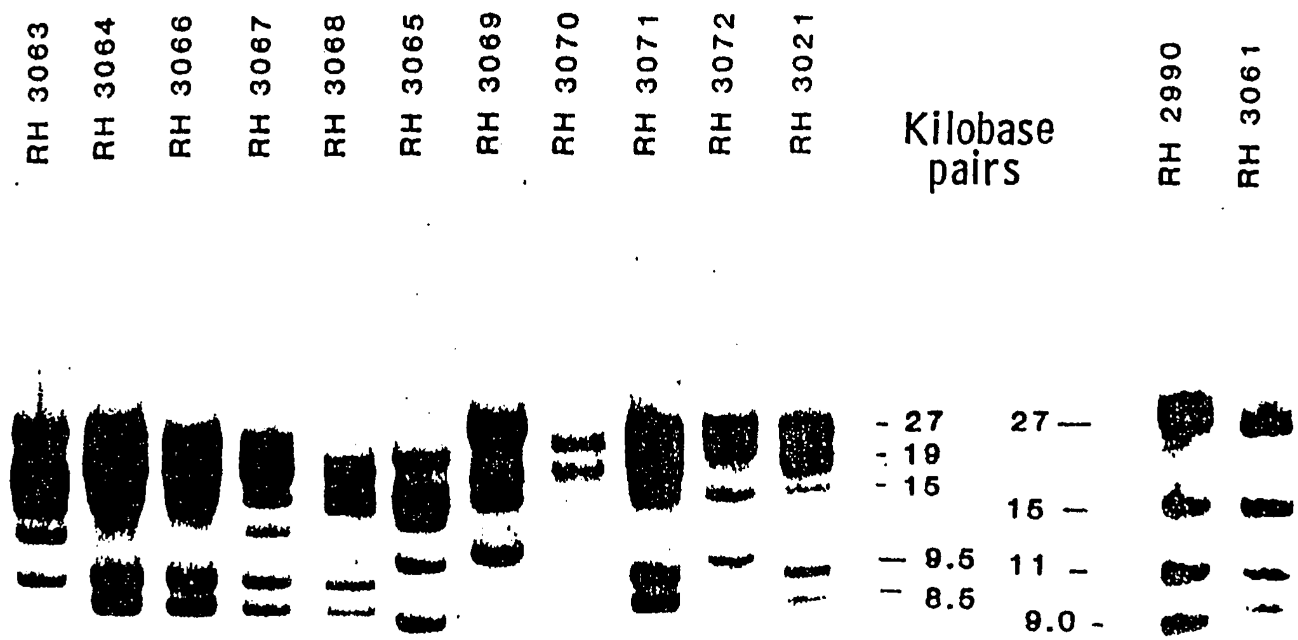
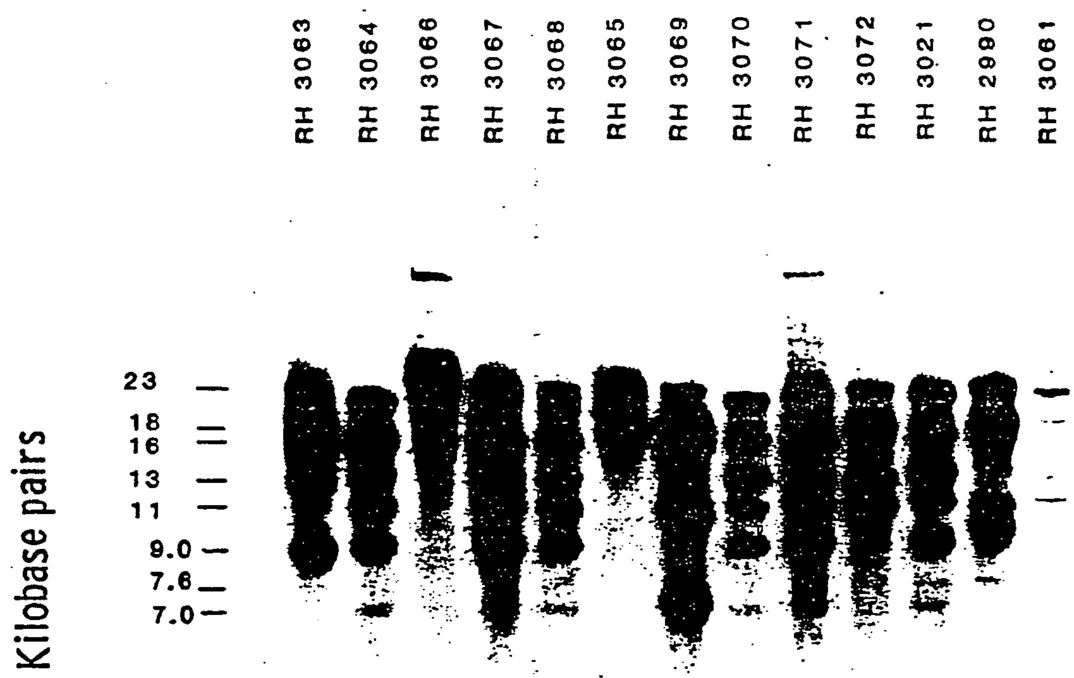


FIG. 7 Bgl II digested DNA from Bacillus subtilis strains



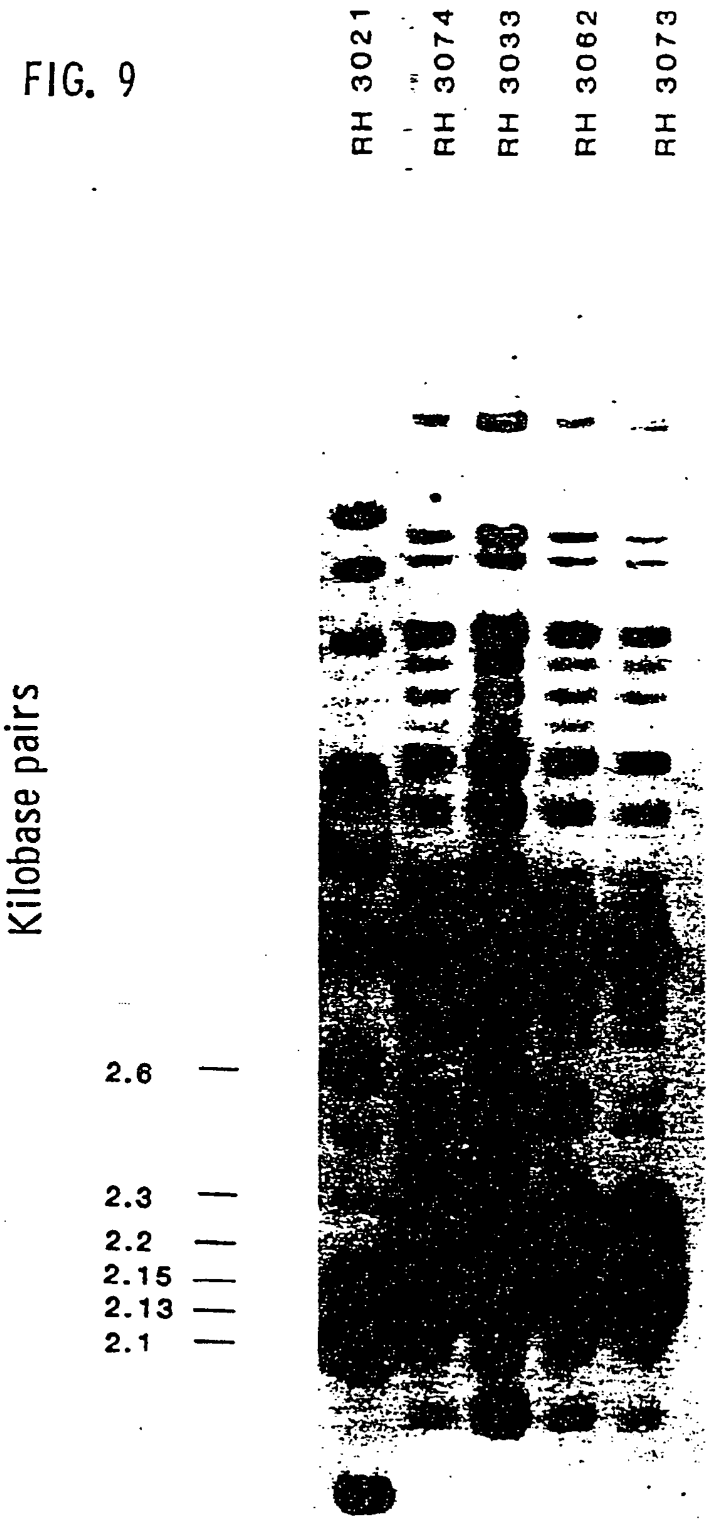
Sac I digested DNA from Bacillus subtilis strains

FIG. 8



EcoR I digested DNA from B. subtilis RH 3021 and B. polymyxa RH 3074, RH 3033, RH 3062, RH 3073

FIG. 9



PST I digested DNA from B. subtilis RH 3021 and B. polymyxa RH 3074, RH 3033, RH 3062, RH 3073

FIG. 10

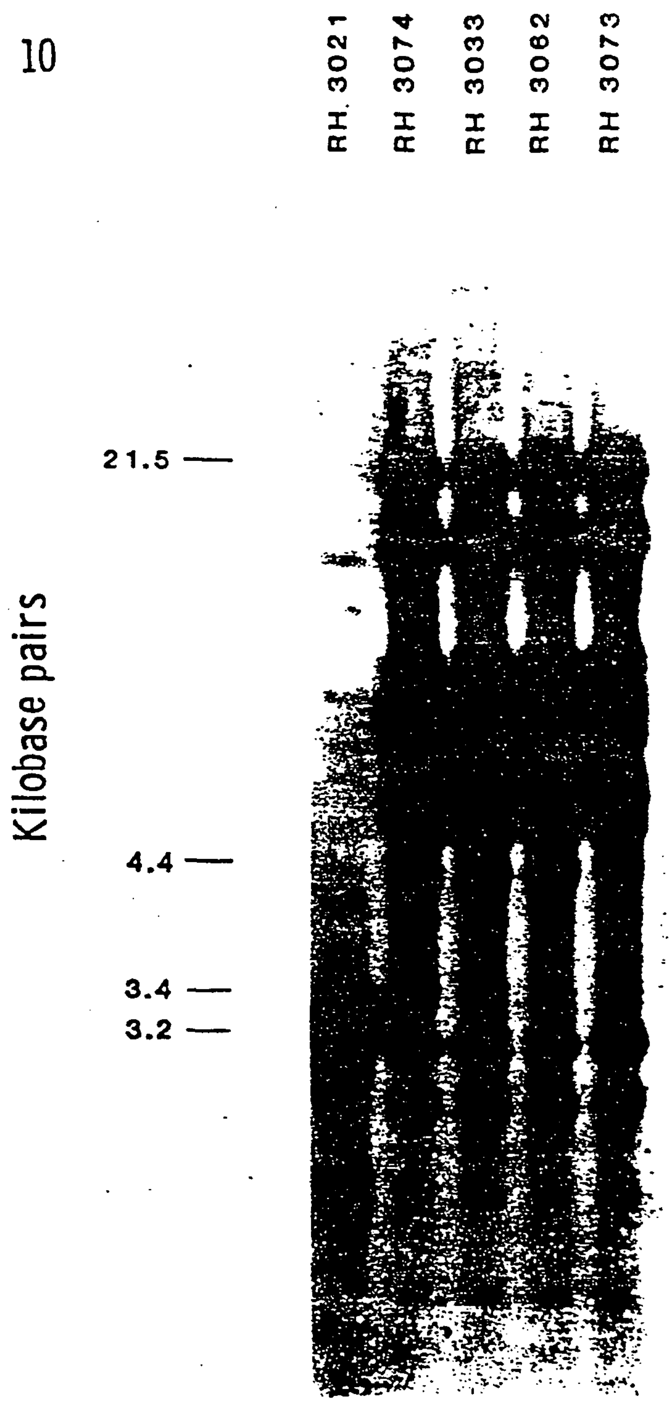
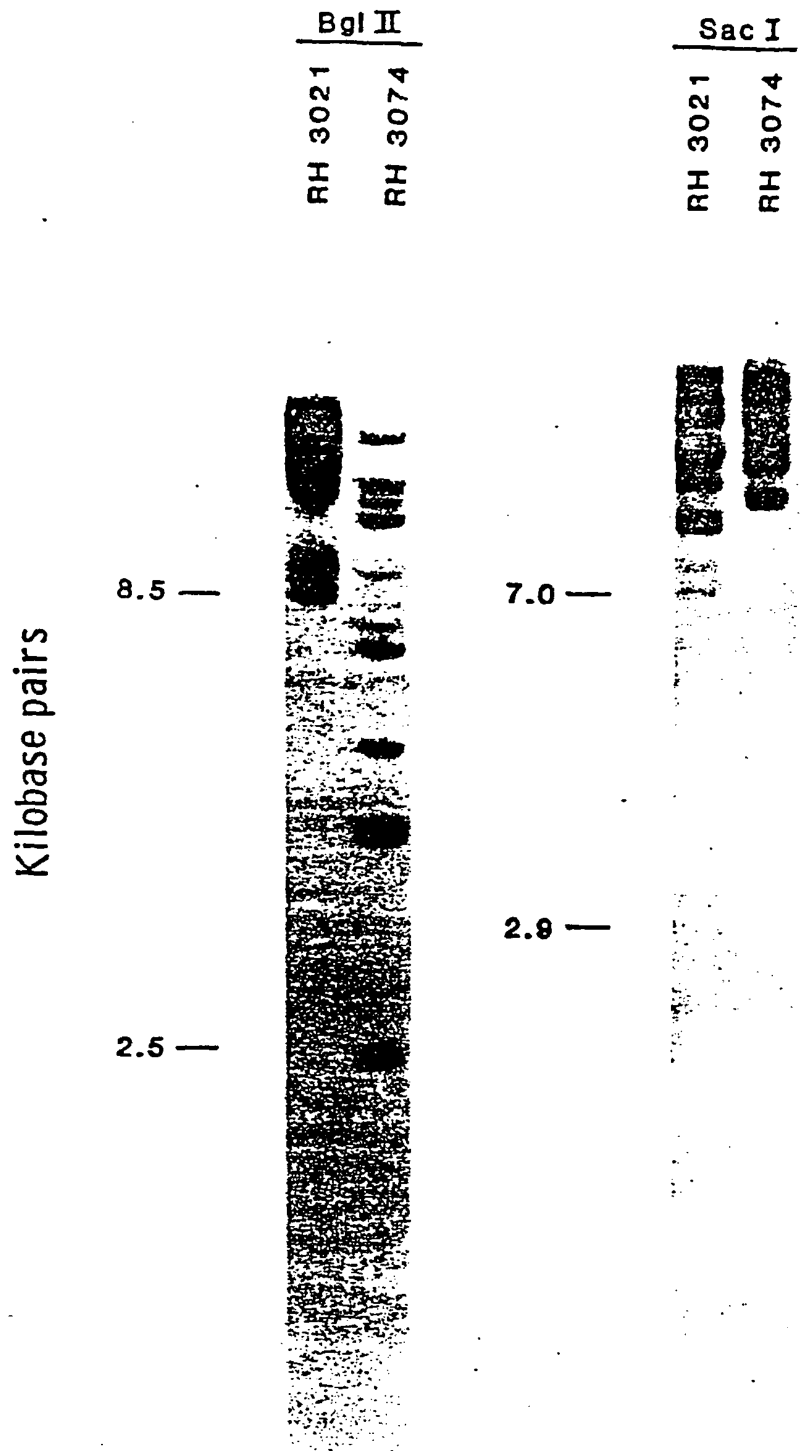


FIG. 11 Bgl II and Sac I digested DNA from *B. subtilis* RH 3021 and *B. polymyxa* RH 3074



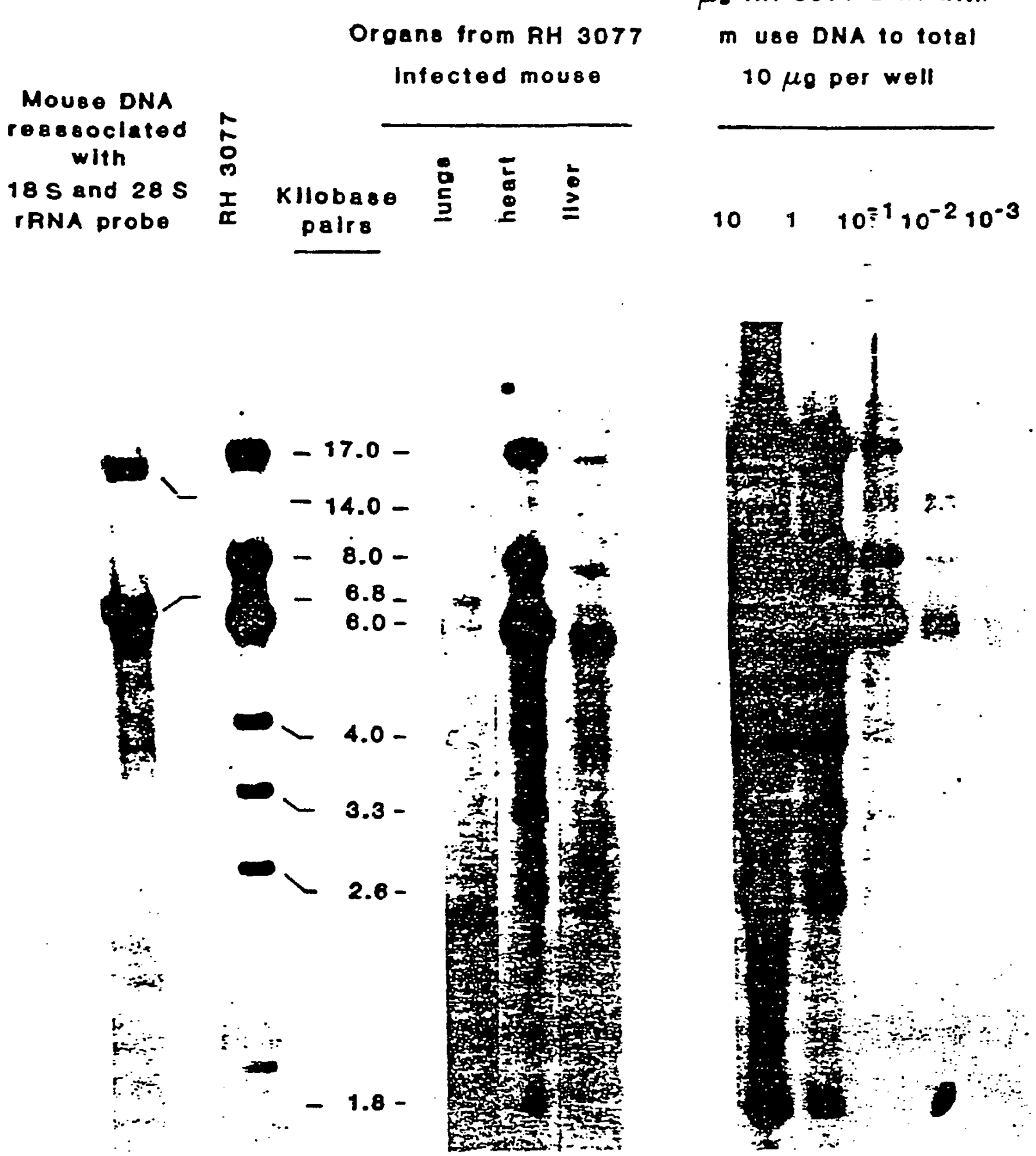
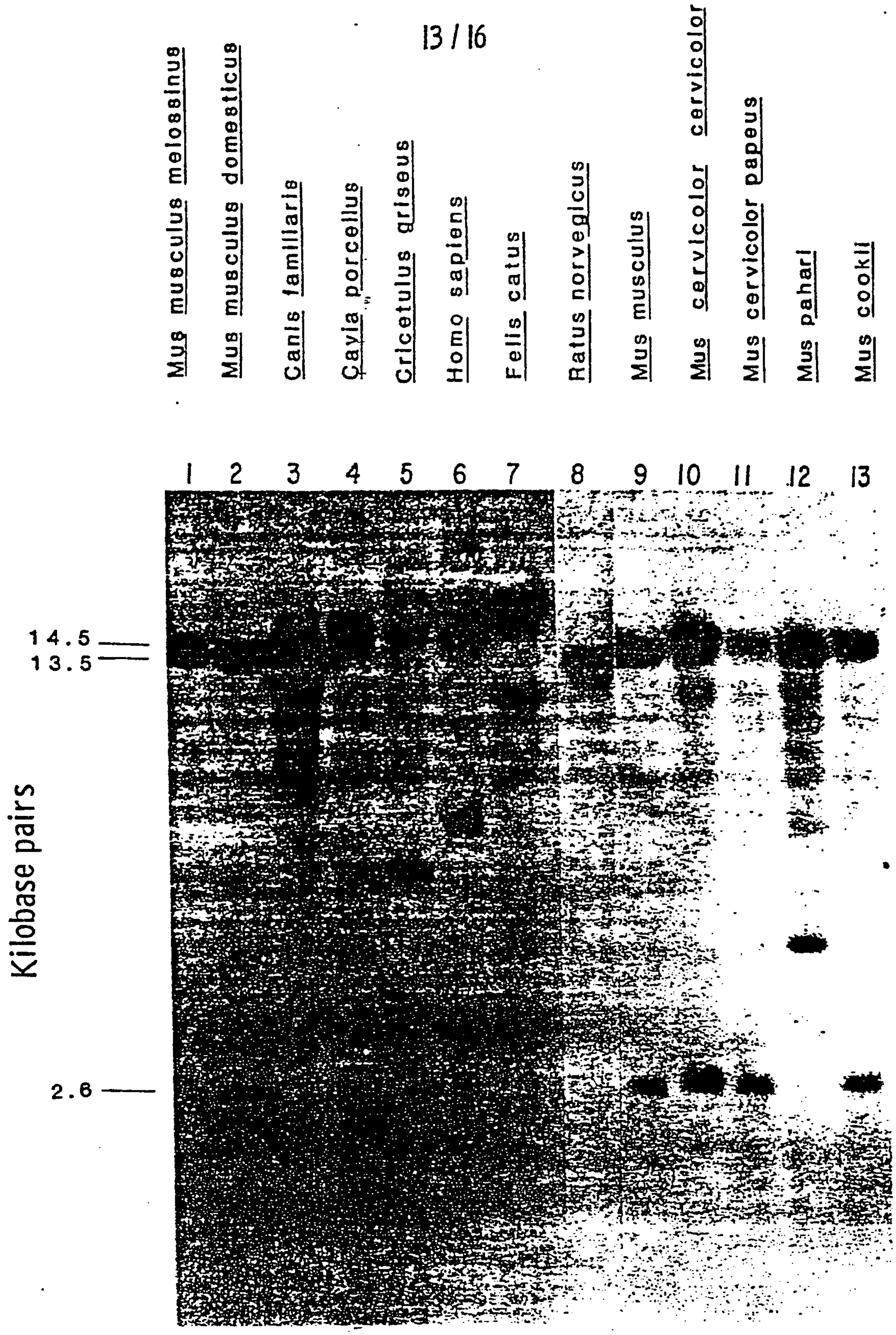


FIG. 12 Detection of S. pneumoniae RH 3077 ribosomal gene sequences in EcoR I digested DNA from infected mouse tissues



Mus musculus melossinus
Mus musculus domesticus
Canis familiaris
Cavia porcellus
Cricetulus griseus
Homo sapiens
Felis catus
Ratus norvegicus
Mus musculus
Mus cervicolor cervicolor
Mus cervicolor papeus
Mus pahari
Mus cookii
1
2
3
4
5
6
7
8
9
10
11
12
13
14.5
13.5
2.6
Kilobase pairs

FIG. 13

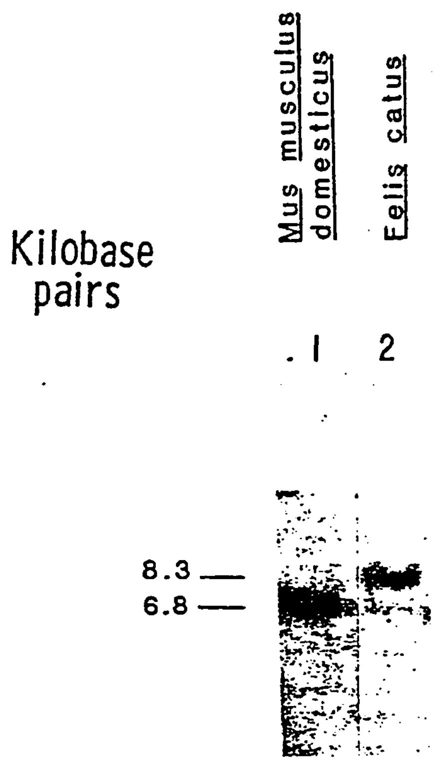

Kilobase
pairs
Mus musculus
domesticus
Felis catus
1
2
8.3
6.8

FIG. 14

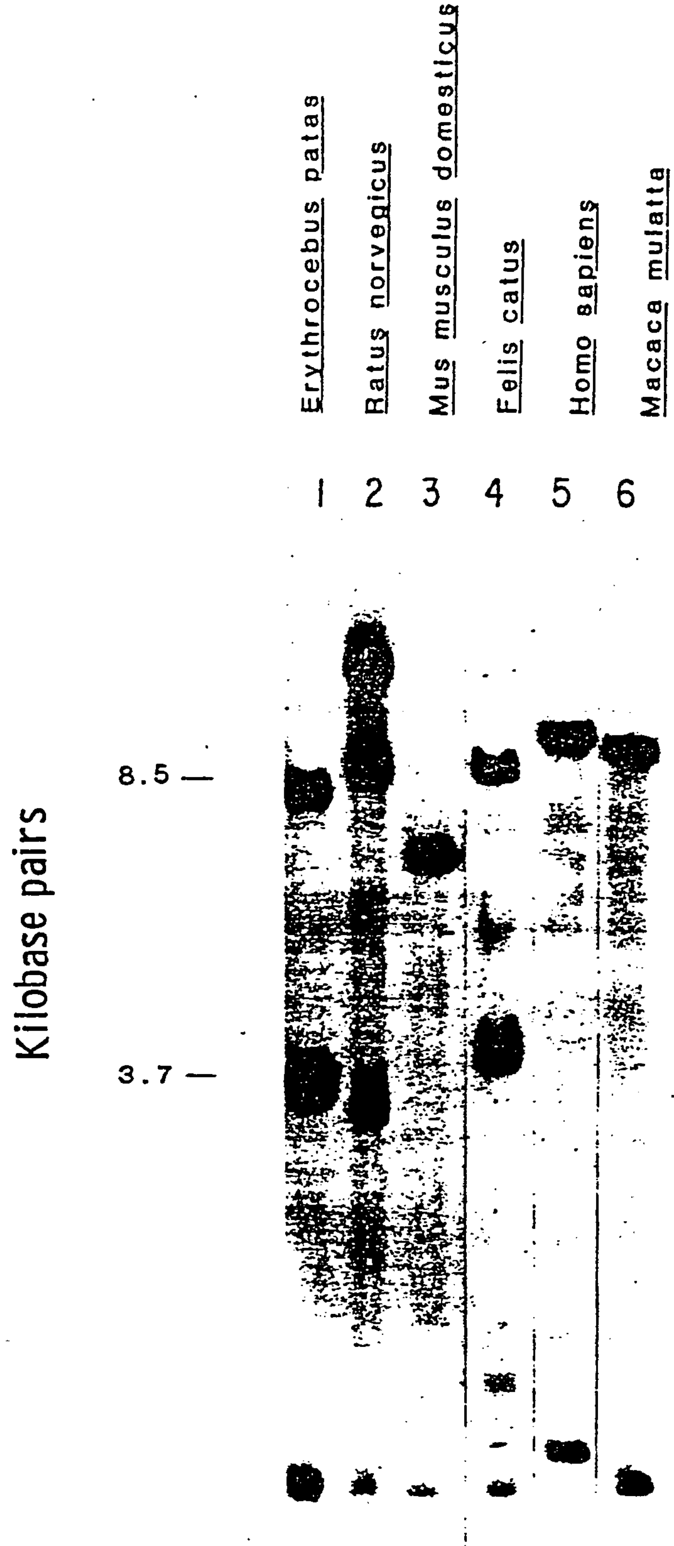

Erythrocebus patas
Ratus norvegicus
Mus musculus domesticus
Felis catus
Homo sapiens
Macaca mulatta
1 2 3 4 5 6
Kilobase pairs
8.5
3.7

FIG. 15

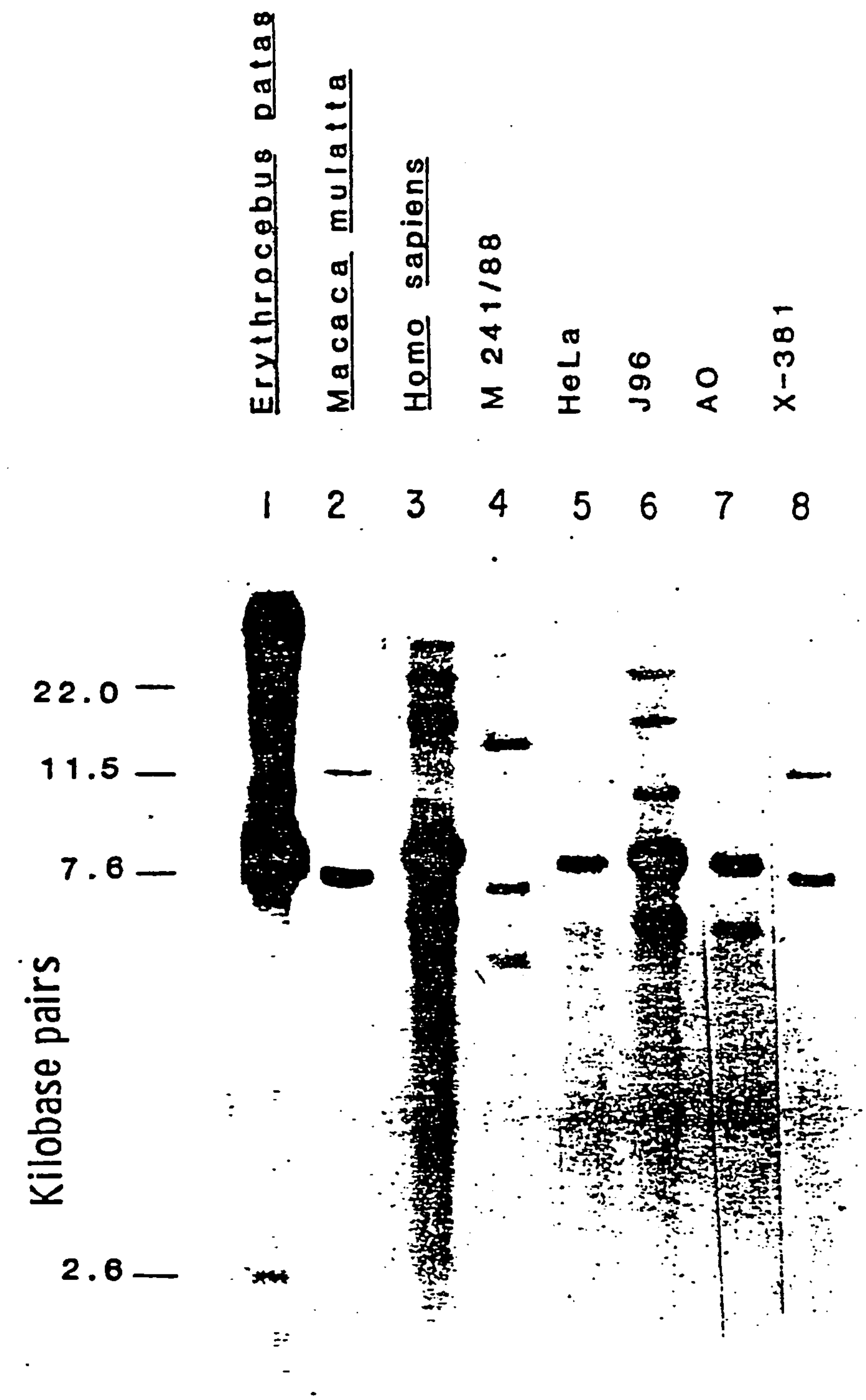
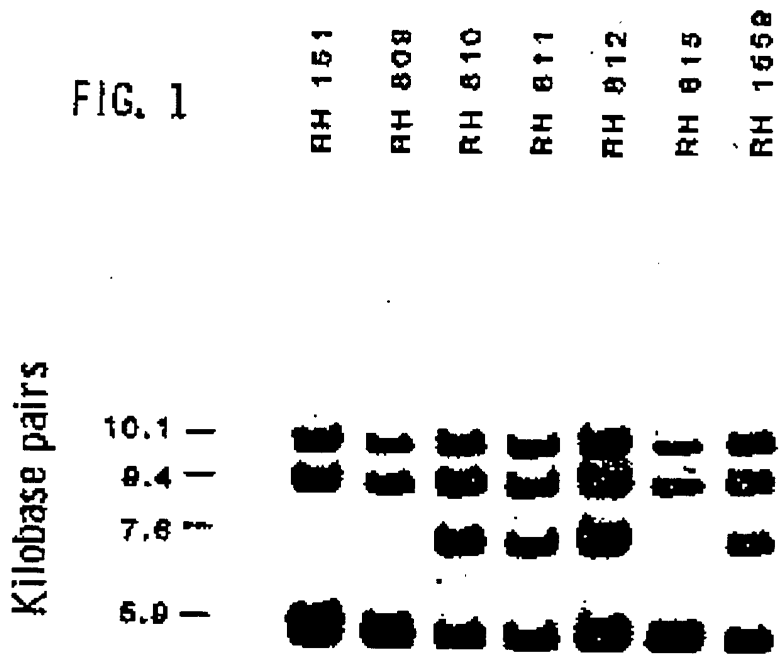

Erythrocebus patas
Macaca mulatta
Homo sapiens
M 241/88
HeLa
J96
AO
X-381
1
2
3
4
5
6
7
8
22.0
11.5
7.6
2.6
Kilobase pairs

FIG. 16

EcoR I digested DNA
from P. aeruginosa strains

FIG. 1



PST I digested DNA
from P. aeruginosa strains

FIG. 2

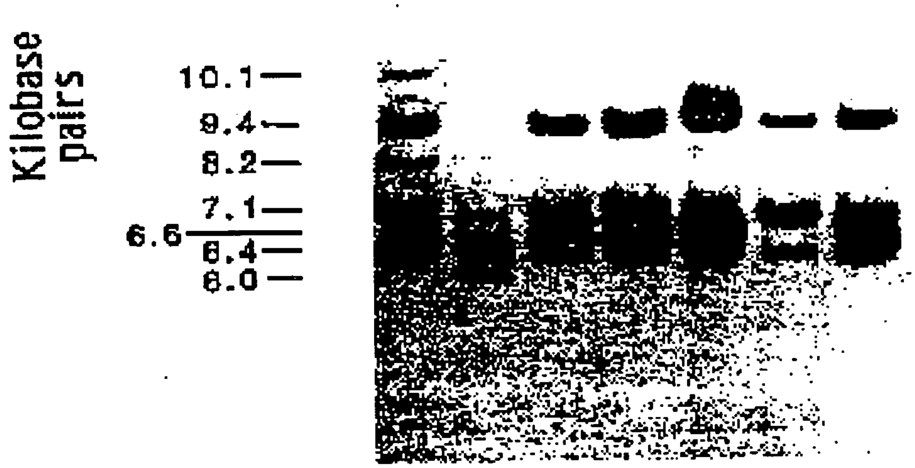


FIG. 3 EcoR I digested DNA from Pseudomonas and Acinetobacter species

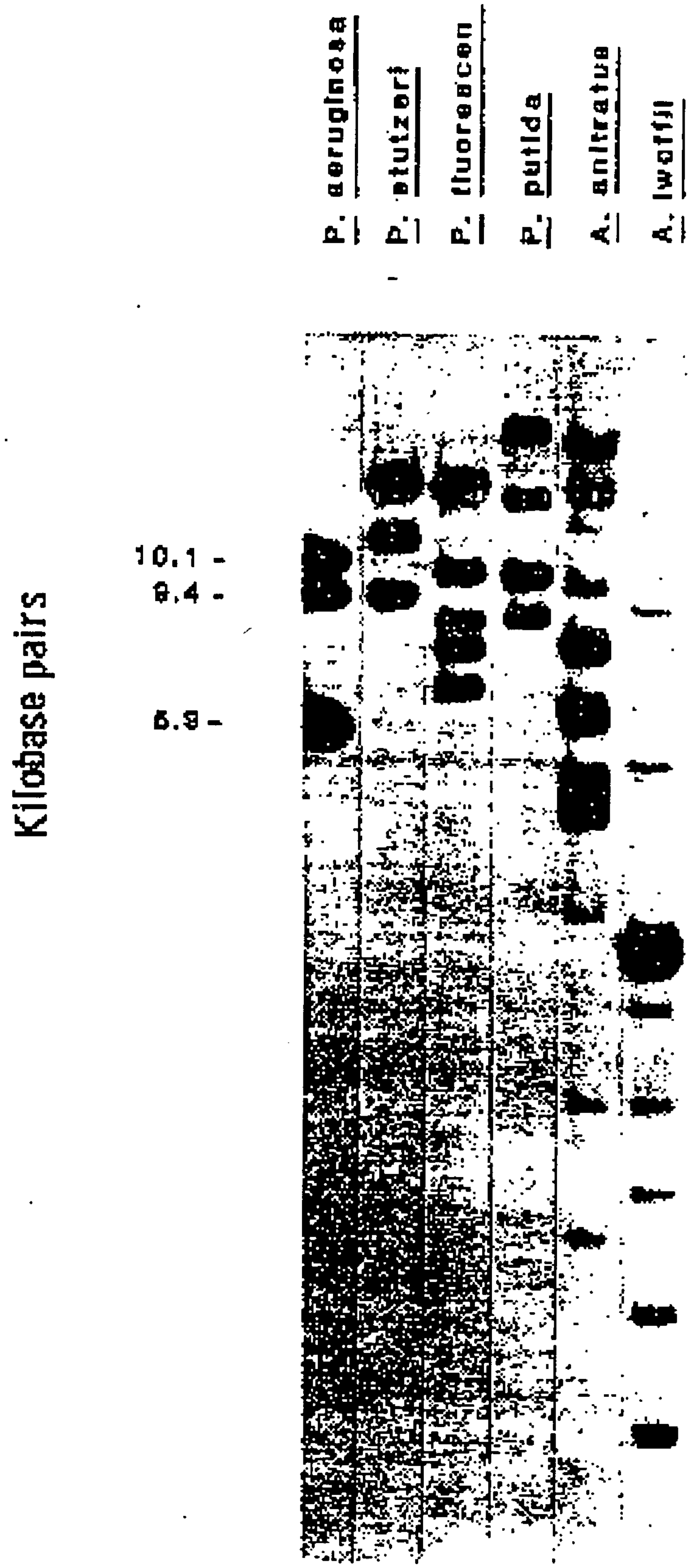
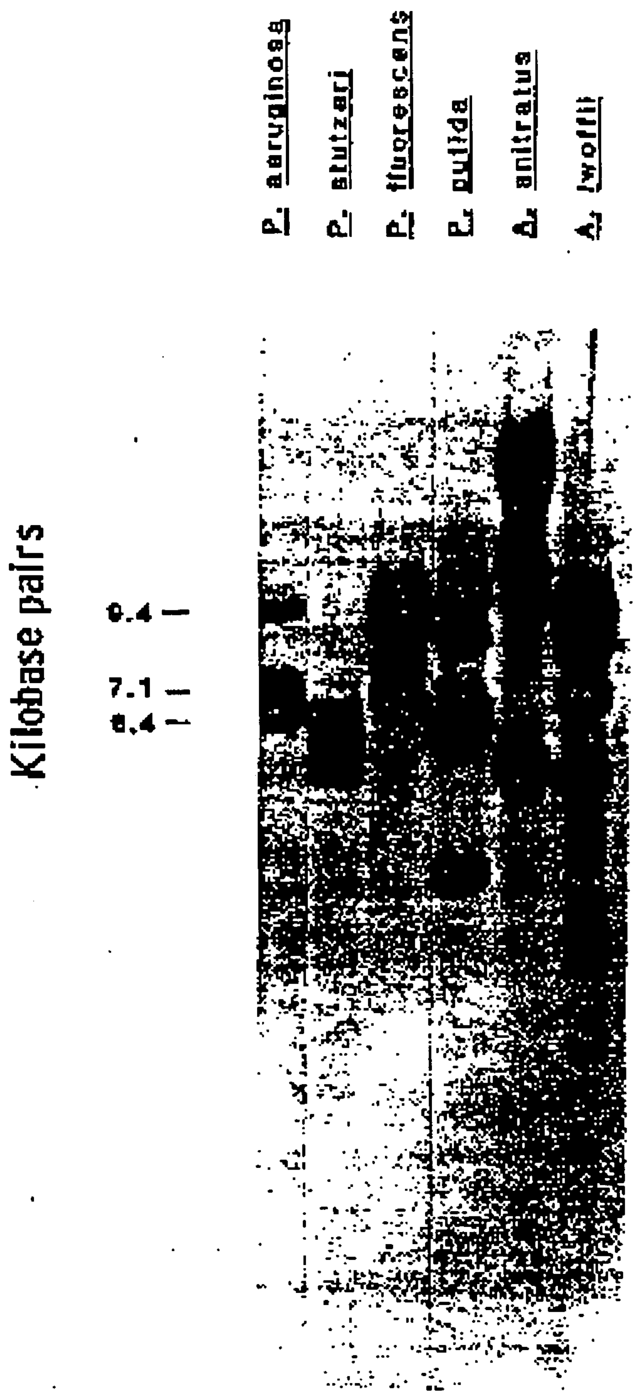
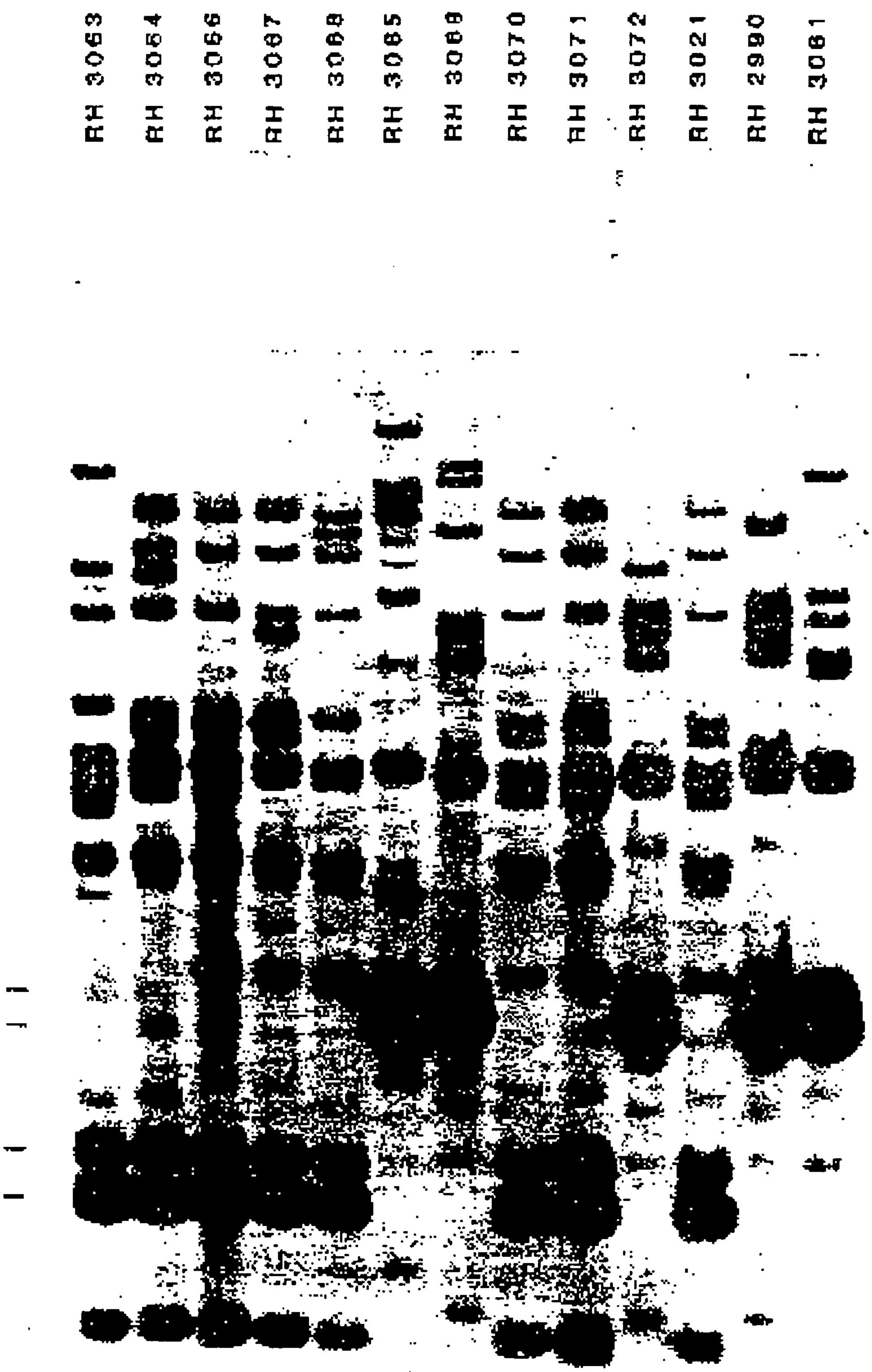


FIG. 4 PST I digested DNA from *Pseudomonas* and *Acinetobacter* species



EcoR I digested DNA from Bacillus subtilis strains

FIG. 5



PST I digested DNA from Bacillus subtilis strains

FIG. 6

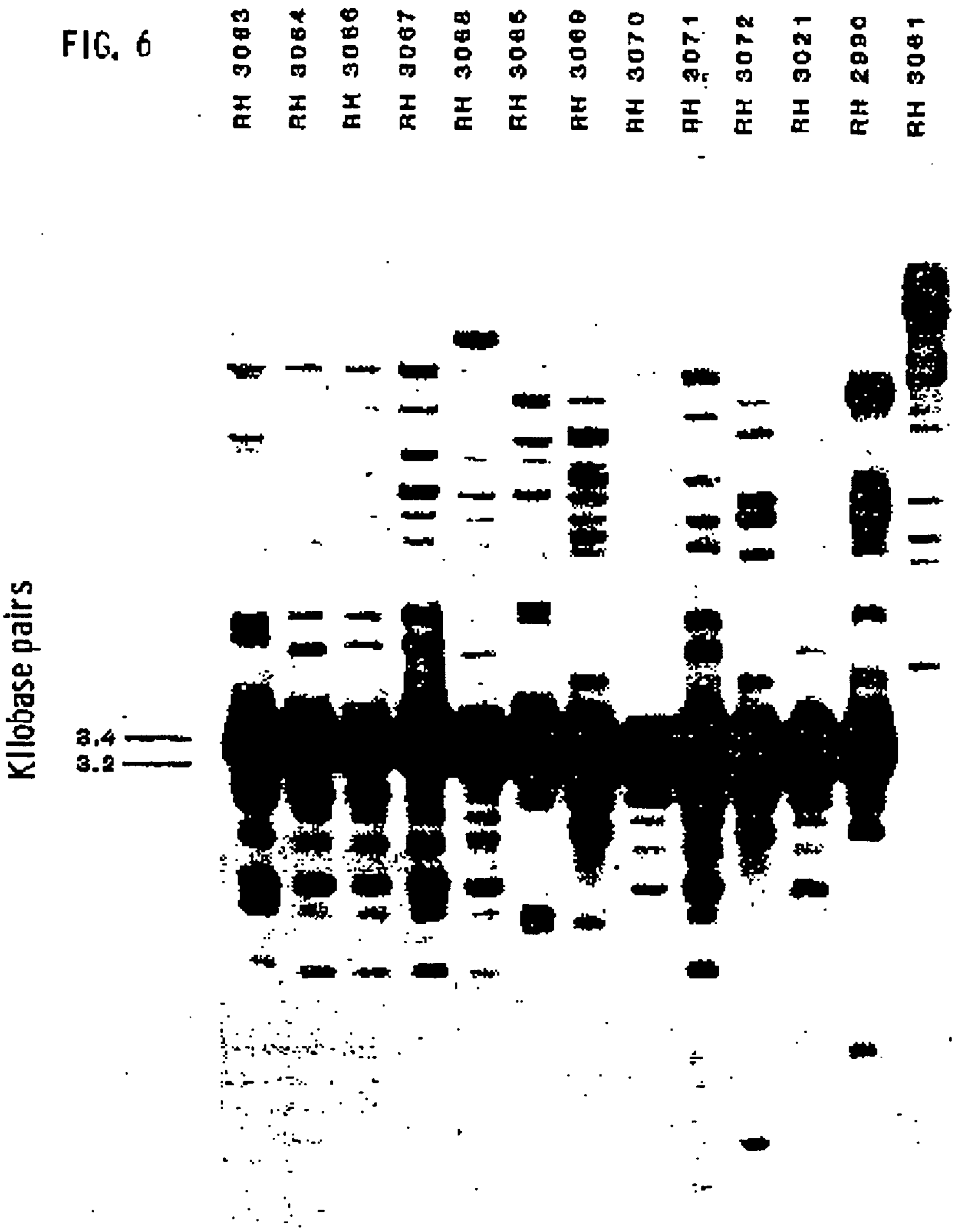
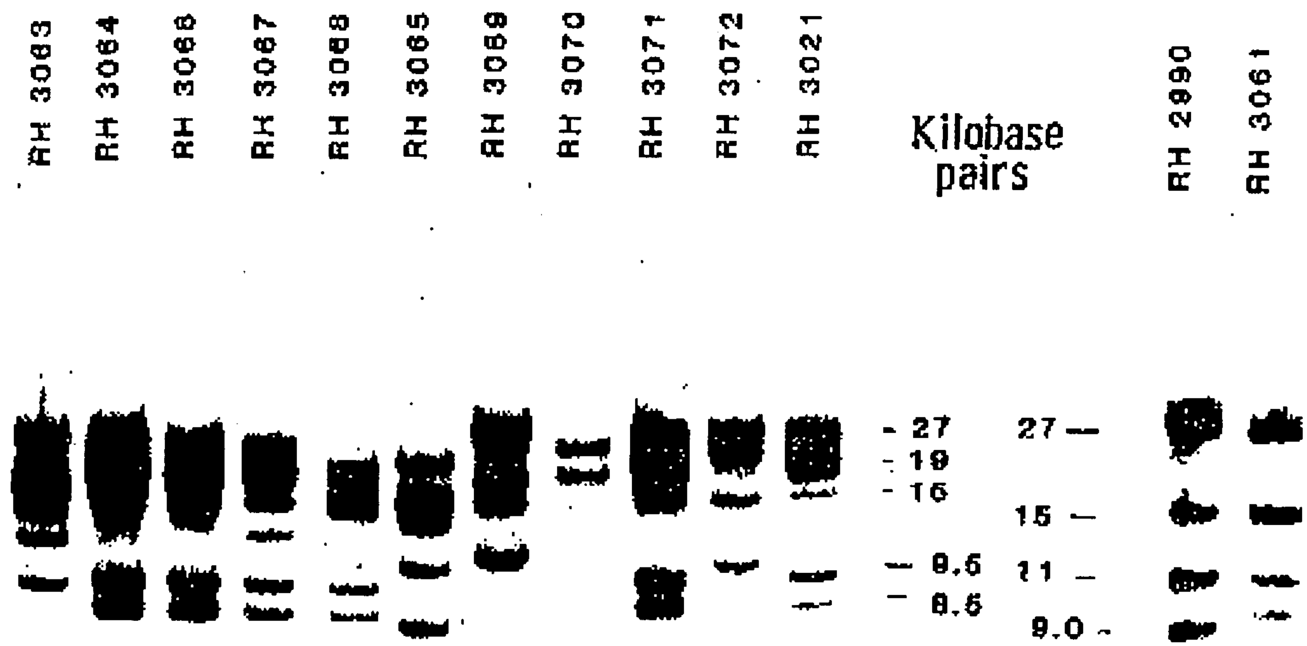
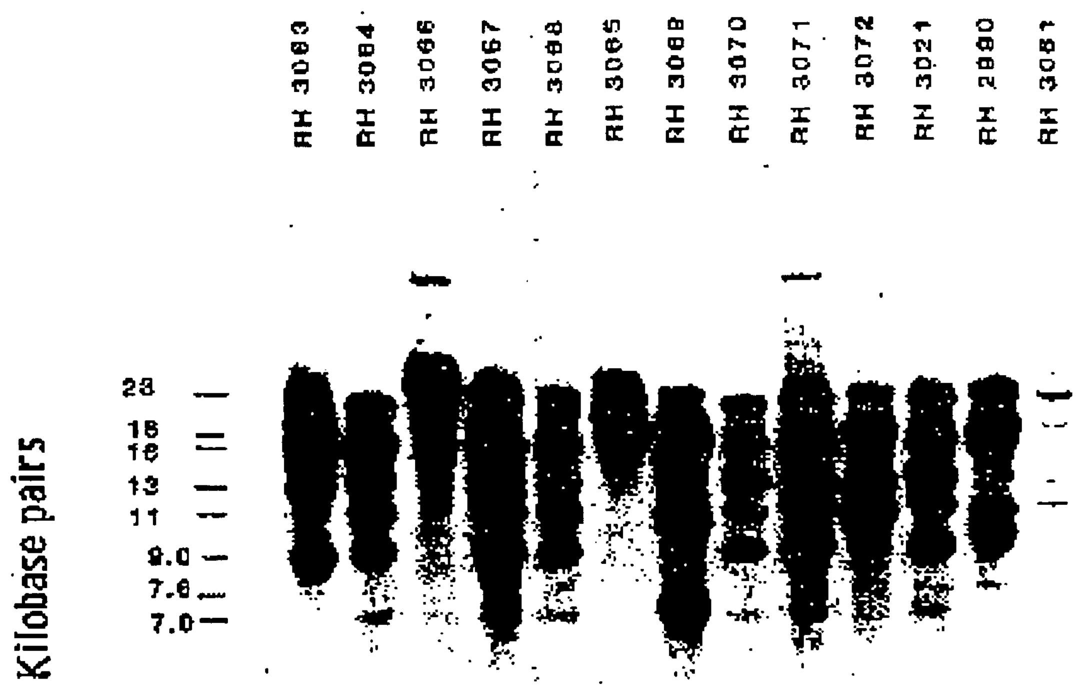


FIG. 7 Bgl II digested DNA from Bacillus subtilis strains



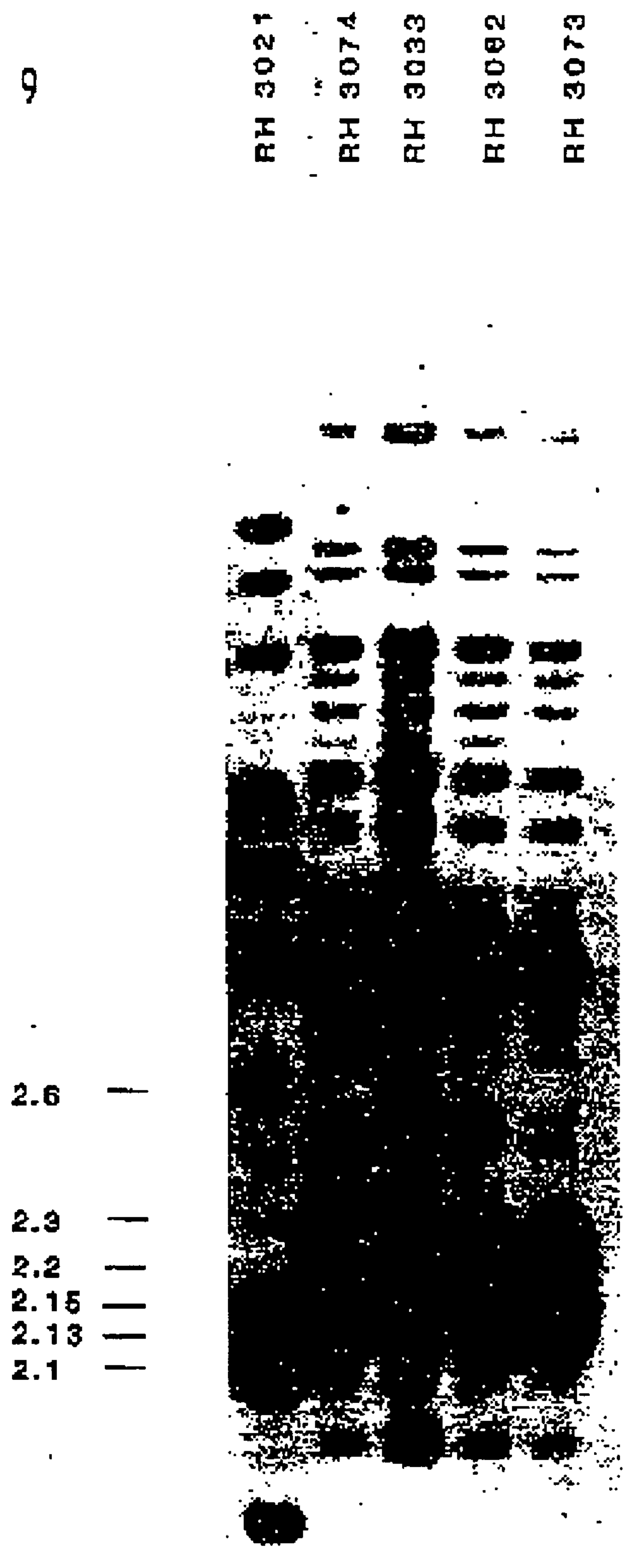
Sac I digested DNA from Bacillus subtilis strains

FIG. 8



EcoR I digested DNA from B. subtilis RH 3021 and B. polymyxa RH 3074, RH 3033, RH 3062, RH 3073

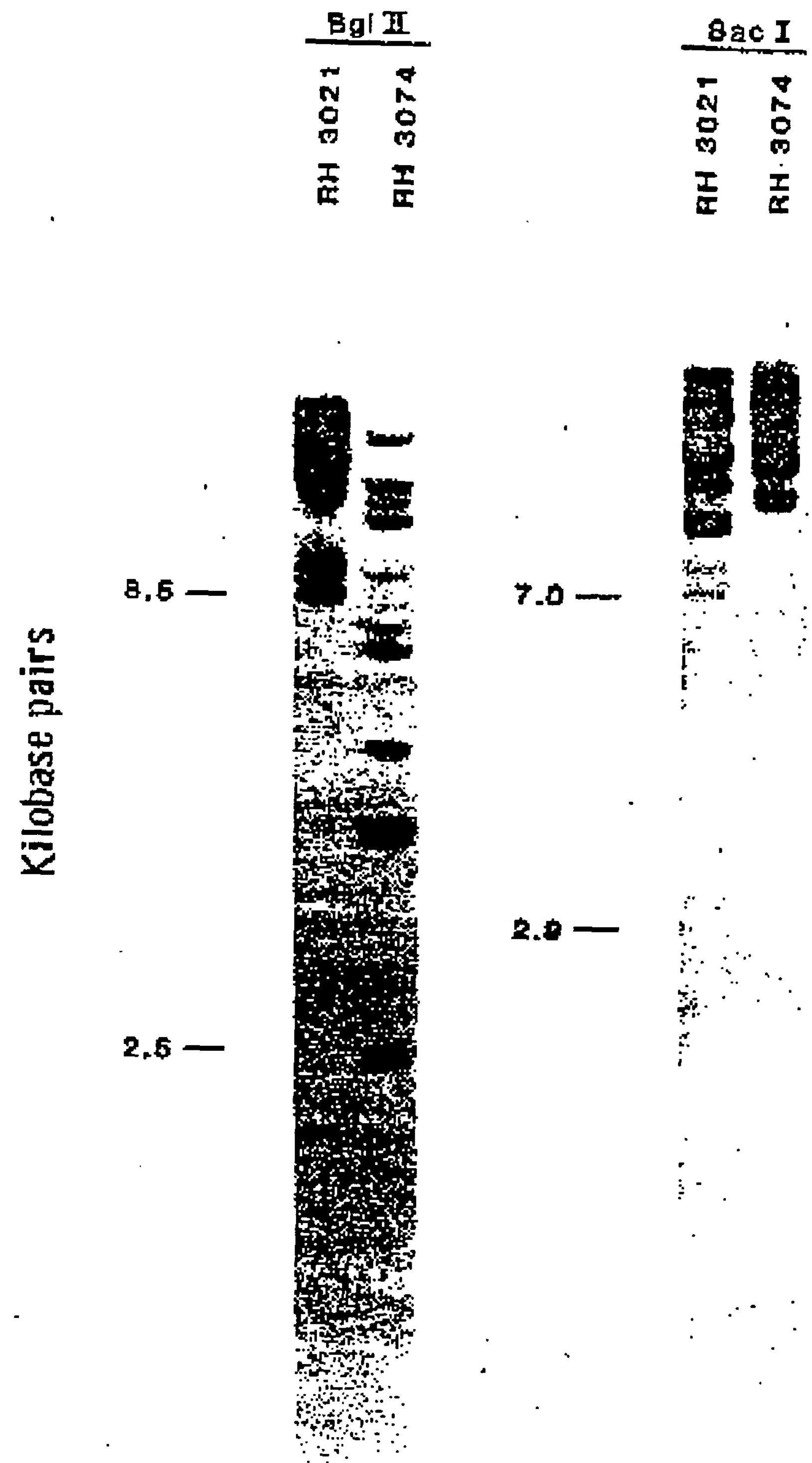
FIG. 9



PST I digested DNA from B. subtilis RH 3021 and B. polymyxa RH 3074, RH 3033, RH 3062, RH 3073

FIG. 10

FIG. 11 Bgl II and Sac I digested DNA from B. subtilis RH 3021 and B. polymyxa RH 3074



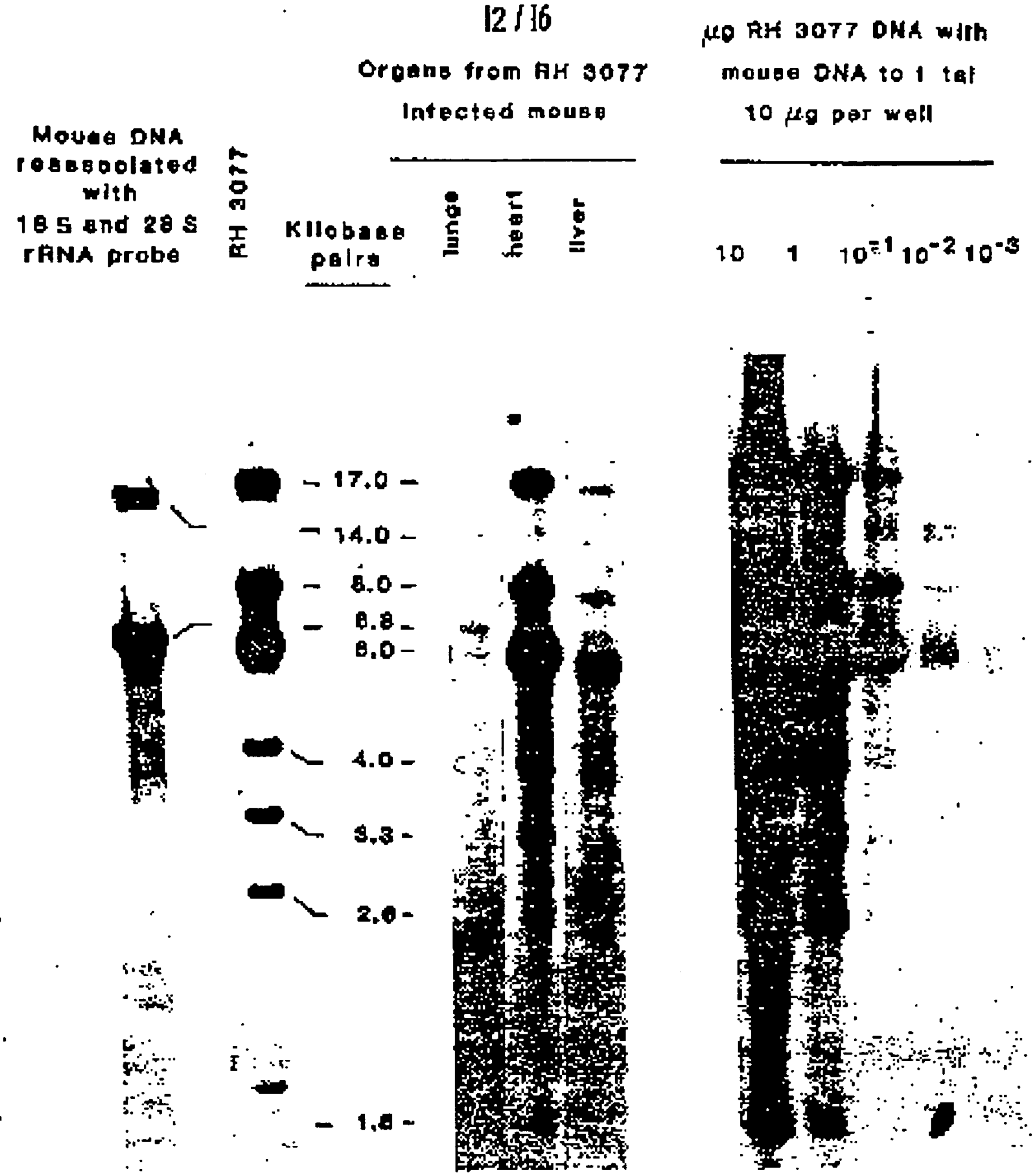


FIG. 12 Detection of S. pneumoniae RH 3077 ribosomal gene sequences in EcoR I digested DNA from infected mouse tissues

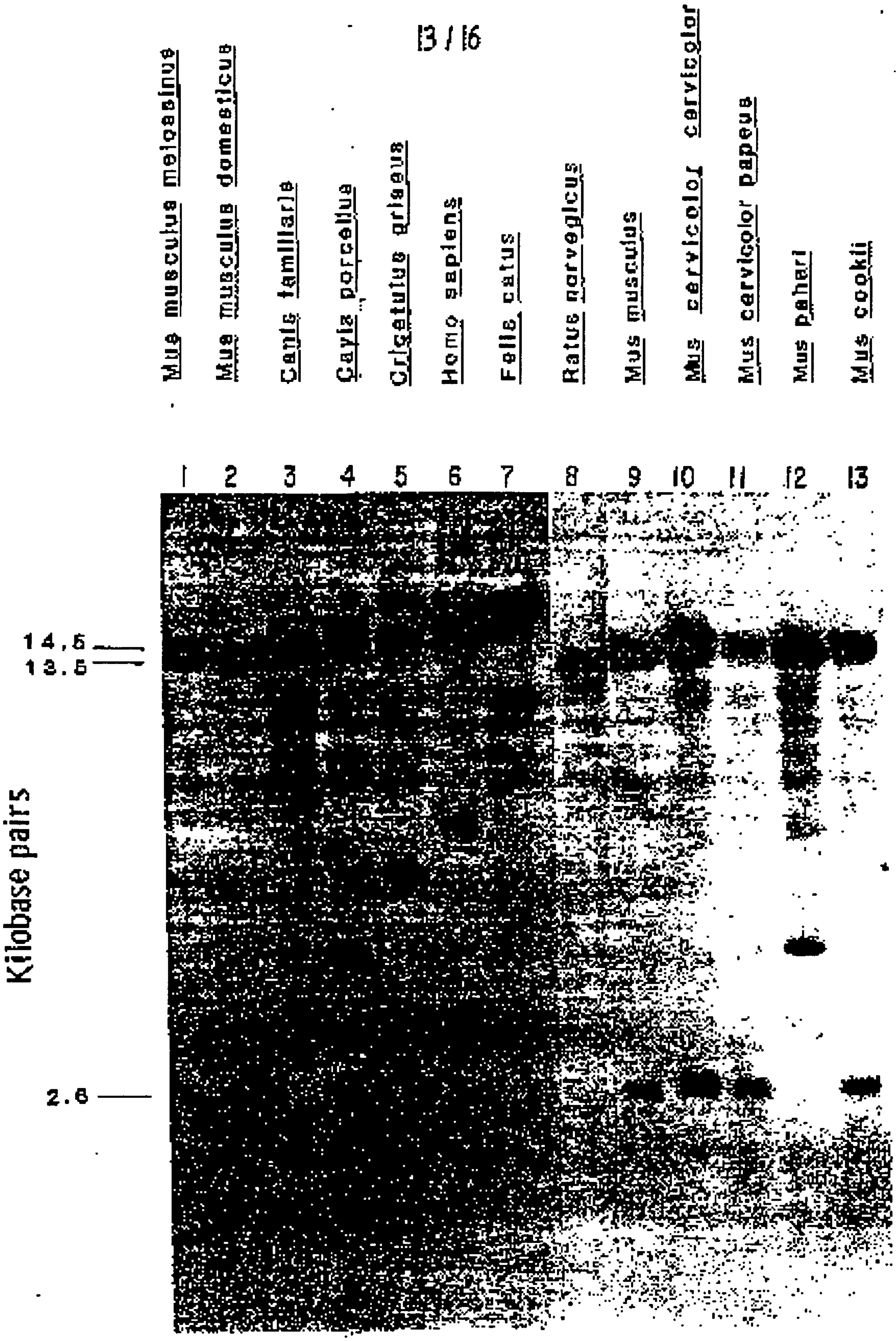

Mus musculus molossinus
Mus musculus domesticus
Canis familiaris
Cavia porcellus
Cricetulus griseus
Homo sapiens
Felis catus
Ratus norvegicus
Mus musculus
Mus cervicolor cervicolor
Mus cervicolor papeus
Mus pahari
Mus cookii
1 2 3 4 5 6 7 8 9 10 11 12 13
14.5
13.5
2.6
Kilobase pairs

FIG. 13


Mus musculus domesticus
Felis catus
Kilobase pairs
1
2
8.3
6.8

FIG. 14

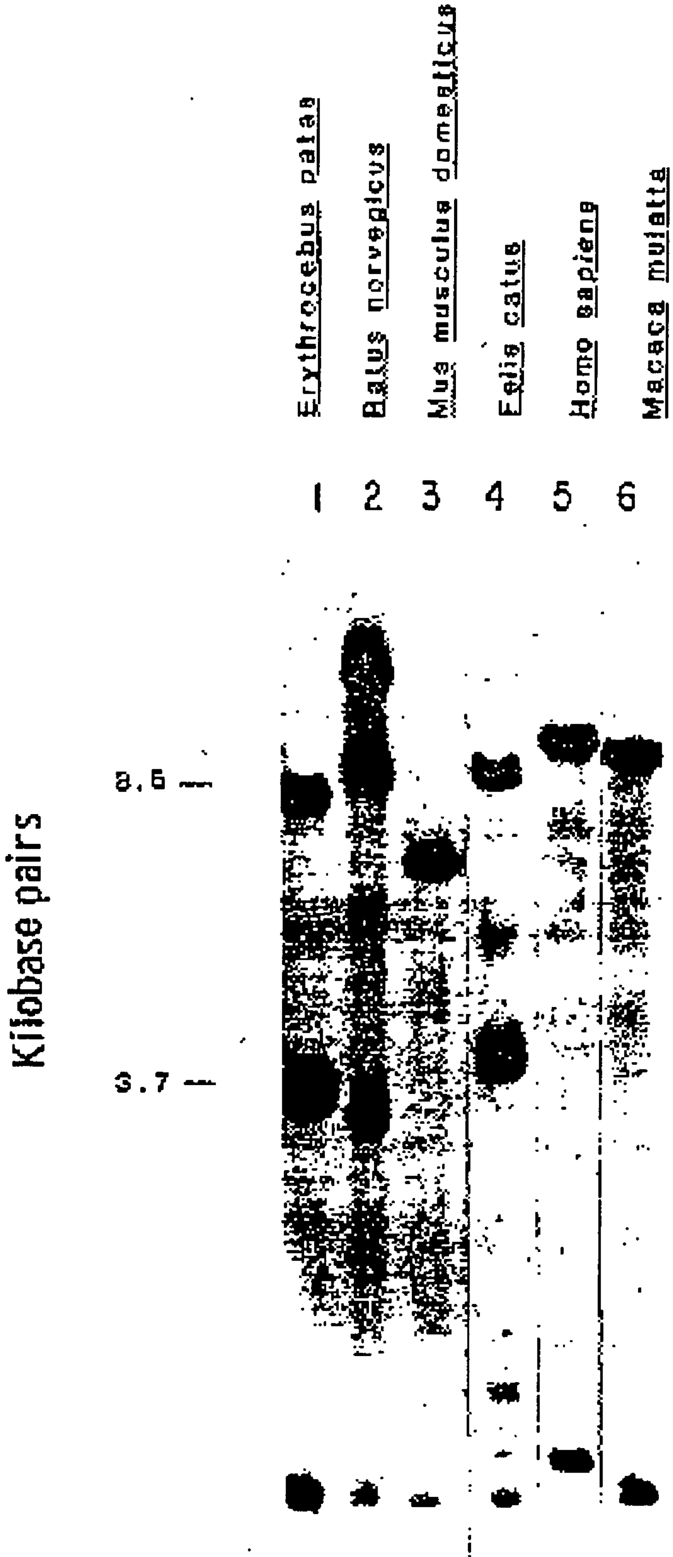

Erythrocebus patas
Rattus norvegicus
Mus musculus domesticus
Felis catus
Homo sapiens
Macaca mulatta
1 2 3 4 5 6
8.6
3.7
Kilobase pairs

FIG. 15

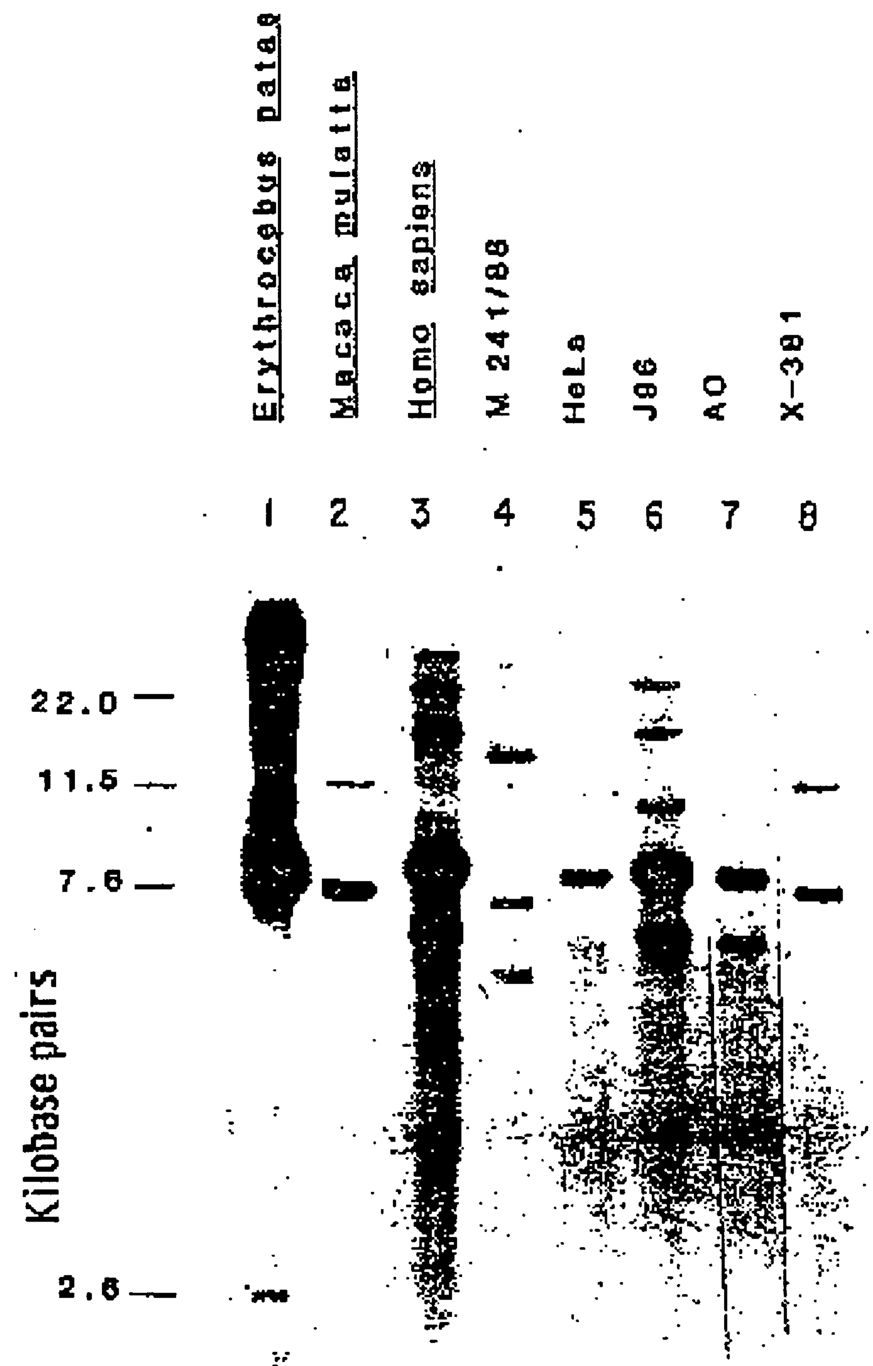

Erythrocebus patas
Macaca mulatta
Homo sapiens
M 241/88
HeLa
J96
AO
X-381
1
2
3
4
5
6
7
8
22.0
11.5
7.6
2.6
Kilobase pairs

FIG. 16